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ON THE BRONCHOCONSTRICTOR EFFECT OF SEROTONIN. BRONCHOGRAPHIC STUDIES ON RABBITS AND GUINEA-PIGS

By E. O. JÄNKÄLÄ and P. VIRTAMA

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(*Received* 24 *October* 1960)

Serotonin is known to be an effective bronchoconstrictor in animals (Herxheimer, 1953; Konzett, 1956; Balzer, Greef & Westermann, 1956). In man a clinical sign of this action is the fact that patients suffering from metastasizing carcinoid tumour often have asthmatic attacks; and Herxheimer (1955) found that although inhalation of serotonin aerosol in four normal cases had no effect, in three out of six asthmatics it elicited severe asthmatic attacks which were abolished by *iso*-propyladrenaline.

The observations made on the bronchoconstrictor action of serotonin have been based on indirect measurements. But it is a straightforward procedure to render the bronchial tree of test animals visible by the methods of clinical bronchography with radio-opaque substances; this technique has therefore been used to demonstrate the effects of serotonin on the bronchi.

METHODS

Twenty rabbits and fifteen guinea-pigs were used. Sixteen rabbits were anaesthetized with urethane intravenously, four with ether; all the guinea-pigs were anaesthetized with ether.

Most of the animals examined received reserpine (7 mg/kg) 24 hr before the experiment in order to abolish the effects of the natural stores of serotonin.

The trachea of the anaesthetized animal was exposed, and a polythene catheter introduced into it. The contrast agent used was Dionosil Aqueous (Glaxo Laboratories, Ltd.). In some experiments (see Table 1) serotonin was administered through the catheter before the injection of the contrast agent; in other experiments serotonin was administered intravenously. After the lungs had been filled with the contrast agent by letting it flow freely into the lungs while the animal was turned about, the trachea was tied and the lungs were removed.

The lungs were radiographed with a Siemens' fine-structure tube equipped with a copper anode at 30 kVp, with Crystallex (Kodak) fine-grain X-ray film developed in D 76 fine-grain developer. This technique allowed a magnification by about ten diameters.

TABLE 1

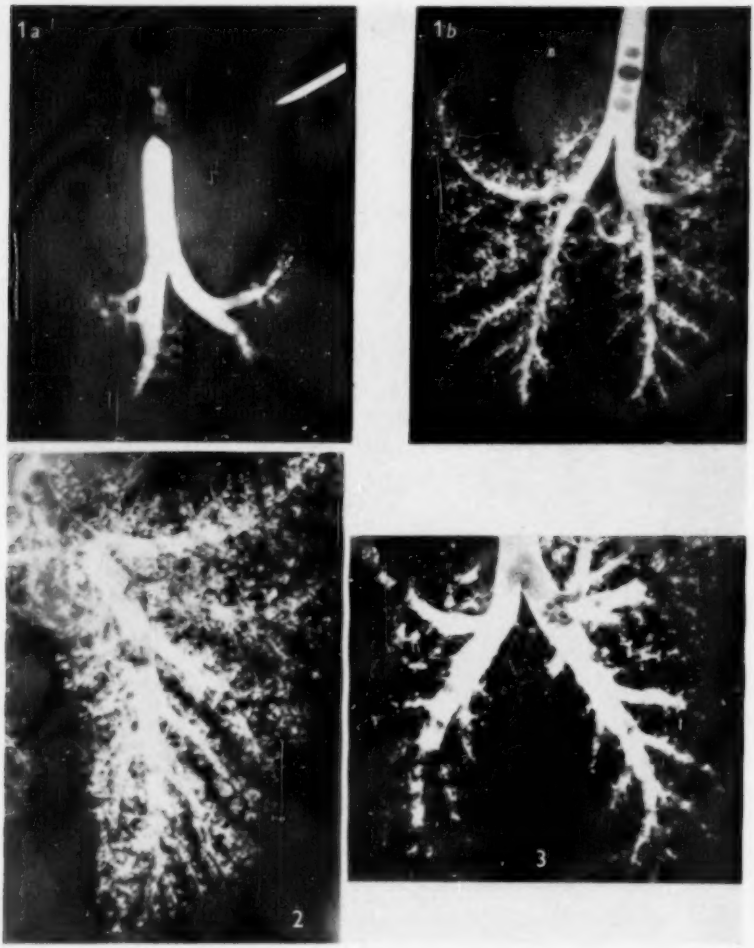
No. and species of test animals used	Premedication, reserpine 7 mg/kg intramusc. 24 hr before expt.	Mode of anaesthesia	Serotonin	Result
2 rabbits	No	Ether	No	Slight bronchospasm
2 rabbits	No	Ether	6 mg/kg I.V.	Slight bronchospasm
3 rabbits	No	Urethane I.V.	No	No bronchospasm
2 rabbits	No	Urethane I.V.	6 mg/kg I.V.	No bronchospasm
3 rabbits	Yes	Urethane I.V.	No	No bronchospasm
4 rabbits	Yes	Urethane I.V.	6 mg/kg endobronch.	No bronchospasm
4 rabbits	Yes	Urethane I.V.	6 mg/kg endobronch.	No bronchospasm
3 guinea-pigs	No	Ether	No	Moderate filling, some bronchospasm
6 guinea-pigs	Yes	Ether	1–12 mg/kg I.V.	Severe bronchoconstriction
6 guinea-pigs	Yes	Ether	1–10 mg/kg endobronch.	Severe bronchoconstriction

RESULTS

Table 1 summarizes the results. The guinea-pigs anaesthetized with ether and the rabbits anaesthetized with urethane or ether differed in their reactions to serotonin. Bronchoconstriction could be readily produced in the guinea-pigs, whether serotonin was given intravenously or endobronchially, and severe bronchospasm occurred (Pl. 1, fig. 1*a*). A normal bronchogram in the guinea-pig is shown for comparison in fig. 1*b*. In the rabbit under urethane, however, no bronchospasm could be evoked whether the animal was pre-treated with reserpine or not, or whether the serotonin was given into the bronchi or intravenously (Pl. 1, fig. 2). In the rabbit under ether a moderate bronchospasm could be induced (Pl. 1, fig. 3).

DISCUSSION

The aim of this work was to render visible the effect of serotonin on the bronchial tree of test animals. In the rabbit under urethane, serotonin had little action whether the animal was pre-treated with reserpine, to deplete its natural stores of serotonin, or not. Geiger & Alpers (1957) have shown that the contraction of smooth muscle by serotonin is antagonized by urethane; this may well explain our results on the rabbit under urethane. In the two experiments where ether anaesthesia was used in rabbits, serotonin given endobronchially or intravenously caused some, though only moderate, bronchoconstriction. The lungs of the rabbit contain relatively high amounts of serotonin. This could make the bronchi rather resistant to serotonin administered. The natural stores of serotonin in the lungs of


1a
1b
2
3

guinea-pigs are lower than those of rabbits (Weissbach, Waalkes & Udenfriend, 1957). In accordance with this fact was the striking bronchoconstriction caused by endobronchially or intravenously administered serotonin.

The bronchoconstrictor action of serotonin could thus be clearly demonstrated radiographically, and to some extent localized in the respiratory tract. The fact that it could be readily seen in etherized guinea-pigs, and only poorly or not at all in anaesthetized rabbits, conforms with the results of experiments with indirect methods by other authors (Schneider & Yonkman, 1954; Konzett, 1956).

SUMMARY

Reactions of bronchi to serotonin were examined with the aid of bronchography in rabbits and guinea-pigs. Serotonin had little effect on the bronchi of rabbits. On guinea-pigs the bronchoconstrictor effect was striking.

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EXPLANATION OF PLATE

Fig. 1. (*a*) Severe bronchoconstriction in a guinea-pig under ether, induced by intravenously administered serotonin 1·2 mg/100 g. Reserpine premedication 7 mg/kg. (*b*) Normal bronchography in a guinea-pig under ether. Reserpine premedication 7 mg/kg. No serotonin.

Fig. 2. Good filling, no bronchospasm in a control rabbit under urethane. No premedication. Bronchography with Dionosil Aqueous. Identical pictures were obtained in rabbits injected with serotonin endobronchially or intravenously up to 6 mg/kg.

Fig. 3. Slight bronchospasm in a control rabbit under ether. Reserpine premedication 7 mg/kg. No serotonin.

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DESENSITIZATION IN THE GUINEA-PIG BY ANAPHYLACTIC MICROSHOCK REPEATED AT DIFFERENT INTERVALS

By E. STRESEMANN
From the Asthma-Poliklinik, Free University of West-Berlin, Germany

(*Received* 22 *February* 1961)

After anaphylactic shock dogs remain refractory to the antigen for a few days (Richet & Portier, 1902). In guinea-pigs exposed to anaphylactic microshock (Herxheimer, 1952), sensitivity returns after an interval of 3–7 days. The present experiments deal with the influence of varying the interval between repeated shocks on the sensitivity of guinea-pigs, and with the influence of the antigen concentration in the aerosol on the shock.

METHODS

Female guinea-pigs of 250–370 g were used. The animals were fed with both fresh and pellet food. They were sensitized with a 5% aqueous solution of dried egg albumin, which was injected on 3 consecutive days (2 ml. intramuscularly on the 1st and 3rd day, 1 ml. intraperitoneally on the 2nd day). After 3 weeks the animals were shocked for the first time by exposing them to aerosol of a 5% solution of egg albumin, according to the method of Herxheimer (1952). Subsequently the shocks were repeated at intervals of 1, 2, 3–4, or 7 days.

The anaphylactic microshock, which consists of severe asthmatic dyspnoea, develops 30 sec–1 hr after the beginning of exposure, according to the degree of sensitivity of the animals. The period required for its development, the pre-convulsion time, was measured with a stopwatch. The dyspnoea begins with an alteration of the breathing movements and of the respiratory rate. When it becomes severe, the abdomen shows deep inspiratory contractions, sometimes with a slowing of the rate.

In some cases these deep abdominal inspirations are less obvious, and the respiratory rate quickens. In these cases a rhythmical up and down movement of the head occurs, caused by the action of the accessory respiratory muscles of the neck. At this stage the animals often begin to sneeze. When abdominal breathing becomes laboured or the head movements pronounced, the exposure must be interrupted, because otherwise clonic convulsions, collapse and fatal shock will follow almost immediately. Even if the exposure is interrupted at this moment, severe dyspnoea will continue for some time and convulsions may develop, but they seldom end fatally.

If the animals are not fully sensitive, dyspnoea develops more gradually and the arrival of the pre-convulsion point—the point at which the exposure must be ended—is more difficult to judge. For this reason we had two observers in order to reduce the possible error, which, with pre-convulsion times of 30–60 min, may be ± 200 sec. If no shock symptoms appeared after a 1 hr exposure, the animal was regarded as insensitive.

Within 3 weeks after sensitization a fairly uniform sensitivity to egg white developed in almost all animals, pre-convulsion times varying from 30 to 175 sec. In most animals this

level of sensitivity remained stable for 2–3 months, but in some animals the sensitivity still increased during the first 3 exposures. It was assumed that in these animals the full degree of sensitivity was attained only after 5 weeks.

The sensitivity of the animals was expressed in terms of the reciprocal of the pre-convulsion time; and each animal's initial sensitivity was taken as 100, since the animals varied somewhat. The figures for sensitivity are thus calculated as

$$\frac{\text{initial pre-convulsion time}}{\text{test pre-convulsion time}} \times 100.$$

Tables 1 and 2 showing these figures include the number of animals in the experiments (n), the mean reciprocal values of the sensitivity ($\bar{y}$), and the standard error of the mean (S.E.). The expected means for the sensitivity are given by the trend lines on Fig. 1. Their calculation is based on the assumption that with equal intervals between exposures sensitivity decreases at an exponential rate: $E(\bar{y}) = a + bt$; $E(\bar{y})$ is the logarithm of the expected value on the trend line, t the time in days which have passed since the first experiment ($t = 0$), a and b are constants. As for $t = 0$ the expected value is 100 and its logarithm is 2, only the parameter b remains to be defined, which has been done according to the method of least squares.

The influence of the concentration of antigen on the pre-convulsion time was investigated by using the method of Armitage, Herxheimer & Rosa (1952). A standard antigen concentration (egg albumin 5%) was used as control and compared with the effect of other concentrations. Guinea-pigs which had constant pre-convulsion times when shocked at weekly intervals were exposed first to the standard concentration, then, after one week, to a new concentration, then, after another week to the standard concentration again, and so on. The influence of any new concentration was calculated from the formula $x = 100\,(1 - C/T)$, where C is the mean of 2 control pre-convulsion times, T the pre-convulsion time of the new concentration under investigation and x the effect of this concentration on the shock as a percentage of that of the standard concentration. In these experiments the variation for the pre-convulsion times in the animal groups was, for unknown reasons, less than in the experiments with varying intervals between exposures. A statistical evaluation for this variation was therefore omitted and the calculation based on the mean pre-convulsion times of the groups.

RESULTS

The effect of varying the interva between exposures

With an interval of 1 day between exposures the sensitivity quickly decreases and the animals become practically insensitive on the sixth day (Table 1, Fig. 1). With exposure on alternate days it takes almost double the time to reach this stage, and with exposure every third or fourth day the development becomes somewhat erratic. Desensitization at first progresses steadily, but from the 11th exposure onwards regresses several times—partial resensitization occurring—and even at the 19th exposure desensitization has not been achieved. It is doubtful whether, with continuing exposures at the same interval, desensitization would have been completed in the same way as with daily or alternate day exposure.

When, after desensitization, the exposure intervals are increased to 7 or 14 days, the sensitivity progressively increases again, particularly during

TABLE 1. Sensitivity of guinea-pigs

1-day interval between exposures (mean initial pre-convulsion time of this group: 88 sec, variation 60–140 sec)

Date ...	12.xi.	13.xi.	14.xi.	15.xi.	16.xi.	17.xi.	18.xi.	19.xi.
n	7	7	7	7	7	7	7	7
$\bar{y}$	100·0	49·7	29·7	22·6	15·3	8·1	3·3	2·9
S.E.		8·6	5·7	6·7	5·4	2·5	0·5	0·3

Date ...	26.xi.	3.xii.	10.xii.	17.xii.	24.xii.	31.xii.	7.i.
n	7	7	6	6	6	6	6
$\bar{y}$	19·4	30·7	43·8	44·0	79·7	55·5	76·2
S.E.	4·6	8·2	10·0	10·4	22·0	11·5	12·5

2-day interval between exposures (mean initial pre-convulsion time of this group: 101 sec, variation 45–200 sec)

Date	10.x.	12.x.	14.x.	16.x.	18.x.	20.x.	22.x.	24.x.	2.xi.	9.xi.
n	13	12	13	13	13	13	13	13	13	13
$\bar{y}$	100	70·1	47·1	37·4	26·2	17·2	10·0	3·8	21·2	48·8
S.E.		3·5	4·0	3·0	2·6	1·8	1·7	0·6	6·9	8·1

3- or 4-day interval between exposures (mean initial pre-convulsion time of this group: 117 sec, variation 50–170 sec)

Date	2.iv.	6.iv.	9.iv.	13.iv.	16.iv.	20.iv.	23.iv.	27.iv.	30.iv.	4.v.
n	10	10	10	10	9	10	10	10	9	9
$\bar{y}$	100	101·4	82·4	64·5	65·8	56·6	43·9	40·4	22·8	26·3
S.E.		11·0	11·9	8·9	9·9	10·2	5·2	7·0	5·0	5·2

Date	8.v.	11.v.	14.v.	19.v.	21.v.	25.v.	28.v.	1.vi.	4.vi.
n	9	9	9	8	8	8	8	8	8
$\bar{y}$	50·0	40·9	29·3	37·0	26·0	18·2	18·6	22·0	25·4
S.E.	9·8	8·9	7·0	8·4	6·9	4·8	5·7	7·3	9·6

7-day interval between exposures (mean initial pre-convulsion time of this group: 97 sec, variation 65–120 sec)

Date	12.xi.	19.xi.	26.xi.	3.xii.	10.xii.	17.xii.	24.xii.	31.xii.	7.i.
n	10	10	10	10	10	10	10	10	10
$\bar{y}$	100	90·4	99·0	93·8	94·7	100·0	94·5	131·2	101·5
S.E.		6·8	8·6	9·4	15·1	11·8	5·6	17·7	14·8

7-day interval between exposures (old animals) (mean initial pre-convulsion time of this group: 180 sec, variation 115–390 sec)

Date	2.iv.	9.iv.	16.iv.	23.iv.	30.iv.	8.v.	14.v.	21.v.	28.v.
n	11	11	11	11	10	10	10	10	10
$\bar{y}$	100	92·3	86·5	80·2	52·9	81·4	51·3	44·1	45·9
S.E.		14·9	16·4	16·0	11·0	12·8	15·6	8·2	9·3

n, number of animals; $\bar{y}$, mean reciprocal values of the sensitivity; S.E., standard error of mean.

the first fortnight. After 5 weeks it has almost reached its original level, indicating that resensitization is now complete.

If exposures are repeated at weekly intervals, the sensitivity remains unchanged. This holds good, however, only for animals which have been sensitized recently. Those sensitized 2 or 3 months previously tend to show a gradual spontaneous loss of sensitivity (Table 1, lowest group).

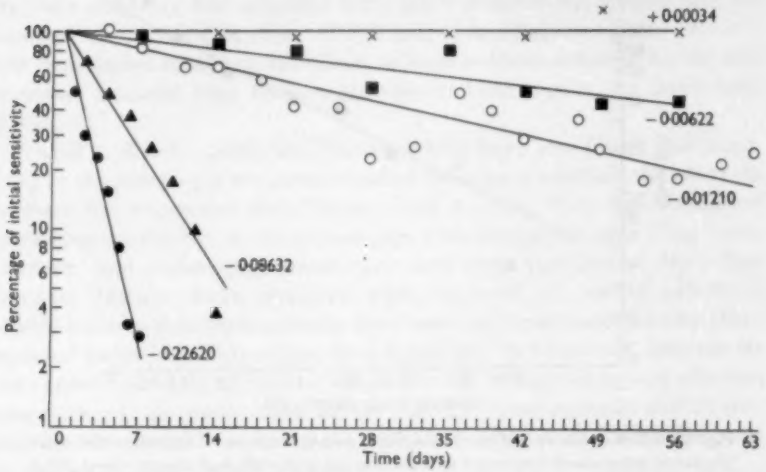

Fig. 1. Effect of varying the interval between exposures: ●, daily exposures; ▲, exposures on alternate days; ○, 3- or 4-day intervals; ×, 7-day intervals; ■, 7-day intervals (old animals). At the end of each regression line is given the value of the constant *b*, the slope of the line. Semi-log. scale.

The individual reactions of the animals vary considerably. There are guinea-pigs whose pre-convulsion times remain practically unchanged for more than 18 months if they are exposed every week. We have observed three such animals out of about 150, whilst in most of the others the pre-convulsion time tends to increase 3 months after sensitization, and in a few this spontaneous desensitization starts immediately after the first exposure to anaphylactic shock. Between these extremes various intermediate reactions exist.

The effect of varying antigen concentration

The antigen concentration in the aerosol had a definite influence on the pre-convulsion time, which is shown with its logarithmic regression line in Fig. 2. In this figure the effect of the standard antigen concentration (5%) is taken as 100%. Lower concentrations had a proportionally smaller effect on the pre-convulsion time. With 0·05% egg albumin there

was little or no dyspnoea in most guinea-pigs, even when the exposure was extended to 1 hr. The highest concentration of egg albumin which could be aerosolized was 7·5%. It shortened the pre-convulsion time, resulting in an increase of effect of 169% of that of the standard solution.

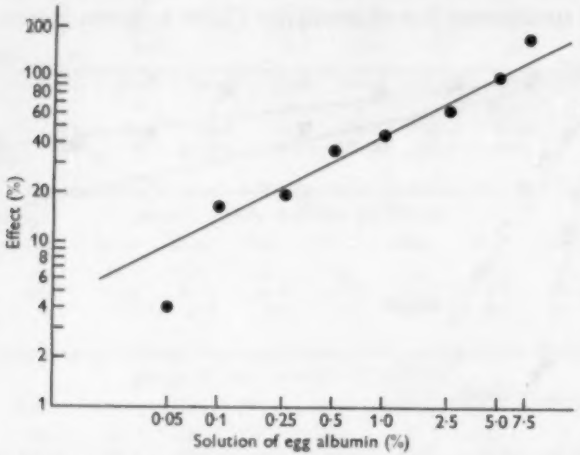

Fig. 2. Effect (ordinate) of various antigen concentrations (abscissa) on the anaphylactic microshock expressed as a percentage of the effect of the standard (5%) solution of egg albumin. Log. scale.

DISCUSSION

In anaphylactic shock the animal's antibody is partly or completely inactivated, depending on the site of introduction and the quantity of the antigen. If a given amount of antigen introduced at weekly intervals elicits an anaphylactic reaction of constant strength, it seems obvious that the amount of antibody present at the time of the reaction is the same. Therefore all antibody removed by the anaphylactic shock must have been renewed within 7 days. Accordingly the production of antibody during any shorter interval is proportionally smaller, leading to weaker anaphylactic reactions; i.e. an antigen introduced at that time will meet less antibody, so that the guinea-pig's pre-convulsion time increases. Eventually, with very short intervals, no antibody or so little will be available that even the largest dose of antigen will no longer produce shock.

These results suggest that not all the antibody present in the animals is equally accessible. It seems as if in the recently sensitized animal a large amount of antibody is superficially located in the bronchial mucosa, and is therefore easily accessible to inhaled antigen. The union of antigen and

antibody can, therefore, occur within a few seconds and shock symptoms supervene before antibody at other sites can be reached by the antigen. At the next exposure this superficially situated antibody is no longer present, and other, less easily accessible, sites will be reached by the antigen which, however, will require a longer time for penetration. On the other hand, it might appear possible that between the first and the second exposure antibody has migrated from the less accessible tissues into the depleted more accessible sites. If this indeed had happened, the amounts thus transferred could not have been as large as those removed by the first exposure, because then the pre-convulsion time would not have been prolonged.

Friebel & Basold (1953) and Friebel (1954) have also found that sensitivity in the guinea-pig was maintained at the same level when the intervals between the exposures were longer than 4 days. They also observed decreasing sensitivity of the guinea-pigs with increasing age. They note, however, that guinea-pigs which were not very sensitive at their first exposure became more sensitive when exposed at weekly intervals. Friebel believes that these animals have been made more sensitive by these repeated exposures. This explanation is unlikely to be correct, because we have never been able to increase the sensitivity of animals by egg albumin inhalation. It is much more likely that for these animals the 21-day sensitization period was too short and their highest antibody titre was reached only after 2 more weeks.

The individual variability of the reaction of the animals in the anaphylactic microshock was considerable. Its cause is probably the individual immunological characteristics of the single animal. The variability is such that the course of sensitization in an individual guinea-pig cannot be predicted with certainty.

SUMMARY

1. The course of the desensitization of guinea-pigs by anaphylactic microshock has been investigated.

2. The shock, as measured by the pre-convulsion time, remains unchanged if exposure to the same amount of antigen aerosol is repeated at weekly intervals. This interval is sufficient for the re-formation of the antibody removed by the previous shock.

3. If the interval is shortened, more antigen is necessary to produce the same shock symptoms: the shorter the interval, the more antigen is required and with short intervals of 24 hr, five consecutive exposures are sufficient to reduce the available antibody so considerably that shock no longer occurs.

4. It is suggested that the antibody is located in different tissue sites, some of which are more easily reached by the antigen than others.

5. With increasing antigen concentration in the aerosol the pre-convulsion time becomes shorter.

I am indebted to Professor H. Herxheimer for valuable help in preparing the manuscript and to Professor K. Freudenberg for the statistical evaluation of the experiments. The technical assistance of Miss G. Karge and Mrs H. Meyer is greatly appreciated.

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RESPONSE OF PACINIAN CORPUSCLES TO SINUSOIDAL VIBRATION

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There has so far been little information regarding the functional significance of Pacinian corpuscles in the body. However, because of their rapid adaptation they must be constantly stimulated by most quick movements of the body, e.g. by heartbeat, respiration and acceleration of the body, and they are also very sensitive to externally applied vibration (Gray & Matthews, 1951*a*). Recently Hunt & McIntyre (1960) have found vibration receptors, which are rapidly adapting, often extremely sensitive and responding to vibratory stimuli between 40 and 700 c/s, in the interosseous membrane of the hind limbs of the cat, and Hunt (1961) has observed similar vibration receptors to those described by Hunt & McIntyre (1960) in close association with the tibial periosteum of the cat. In this study he identified these receptors as Pacinian corpuscles. It is therefore desirable to investigate the response of Pacinian corpuscles to sinusoidal vibration in more detail, and to compare its results with the experiments of Hunt & McIntyre (1960) and Hunt (1961), and also with those on human subjects (Geldard, 1940).

Although in the past many experiments have been carried out with Pacinian corpuscles in the mesentery and in other parts of the cat, there have been only a few experiments dealing with the stimulation of corpuscles by sinusoidal mechanical stimuli (Scott, 1951; Loewenstein, 1958). These, however, do not seem to give much information on this subject. This paper will therefore describe experiments in which the threshold was determined of the mesenteric corpuscle, either isolated from the cat or undissected and with intact circulation, to sinusoidal vibrations of various frequencies. The receptor potential from the non-myelinated ending inside the corpuscle (Gray & Sato, 1953) in response to vibrations has also been investigated.

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METHODS

Preparation. All the experiments were carried out with Pacinian corpuscles of the mesentery. Cats anaesthetized with sodium pentobarbital (Nembutal; Abbott Laboratories) were used. In most of the experiments a piece of mesentery was cleansed of connective tissue and fat under a binocular microscope.

Recording. Recording of impulses was made from the axon, suspended in liquid paraffin overlying the mammalian Ringer's solution, in which the mesentery and corpuscles were immersed. For recording impulses from the corpuscle in the mesentery of the living cat the abdomen of the cat was opened under anaesthesia, and a part of the mesentery, containing a few isolated corpuscles, was stretched on a small block of wood, this being held tightly on supporting bars with a clamp. The axon to the corpuscle under study was dissected from the surrounding connective tissue without disturbing the blood supply, and was suspended in paraffin oil on two platinum-iridium wires connected to the input of the amplifier.

In order to record the receptor potential of the Pacinian corpuscles the corpuscle and its axon were mounted in two saline pools which were separated by an air gap; in most cases the nodal component potentials were abolished by employing 0·5% procaine Ringer's solution (Gray & Sato, 1953). The potential changes between two saline pools were led, through platinum-iridium electrodes, to a capacity-coupled amplifier (Tektronix type 122) and were displayed on a cathode-ray oscilloscope (Tektronix type 532). When the receptor potential was recorded, an over-all time constant of 1 sec for the recording system was generally used.

Stimulation. Mechanical stimulation of corpuscles was produced by a fine glass stylus attached to a ceramic piezo-electric bender element (kindly provided by Gulton Industries, Metuchen, New Jersey), across which potential changes of either sinusoidal form or of rectangular pulse shape were applied. For producing sinusoidal oscillation, an audio oscillator (Hewlett Packard Co. 200AB) was employed over the frequency range of 20–1000 c/s. In the early stage of the experiments the sinusoidal mechanical stimulation was suddenly applied to the corpuscle by opening an output shunt of the oscillator; but at the later stage sinusoidal vibration, which increased gradually to its final amplitude, was employed by modifying the oscillator circuit. The rise time of the amplitude increase varied according to the frequency; it was longer when the frequency was lower. However, this hardly affected the results obtained.

Calibration of the displacement of the stylus. The movement of the piezo-electric bender element under voltages of various magnitudes and frequencies was examined by the following two methods. First it was measured visually, using a microscope giving 430 times magnification. The movement was proportional to the voltage applied to the element up to about 150 V, and the absolute displacement of the glass stylus to a rectangular mechanical pulse of a sufficient duration was found to be about 0·4 μ/V. This is nearly the same as that of a Rochelle salt crystal (Gray & Sato, 1953). The displacement of the bender element to sinusoidal oscillation of various frequencies up to 1000 c/s did not differ very much, but its accurate measurement was rather difficult with the above method. Therefore an alternative method was employed in which a transducer tube (RCA 5734) was mounted on a micromanipulator with a calibrated microscrew. Contact between the transducer and the tip of the glass stylus could readily be decided, as they were approximated by the manipulator. By determining the position of contact with the bender element when unexcited or when driven by the oscillator, the amount of movement could be measured. This was done over the frequency range from 20 to 2000 c/s. The movement was found to be nearly uniform from 20 to 1000 c/s within a 10% error, but there was a sharp increase in the displacement at 250–300 c/s by about 30% (Fig. 1). This may have been resonance introduced by the glass stylus, since the resonance frequency of the bender element is about 2000 c/s. In Fig. 1 a decrease of the movement is seen at the frequency of less than 60 c/s, but this might

have been due to measurement error, because it became difficult to determine the contact between the transducer and the stylus when the frequency was lowered.

Temperature. Most of the experiments on isolated corpuscles were carried out at room temperature of 21–26° C. Some experiments were made *in vivo* on undissected corpuscles with intact circulation. One successful experiment was also made on the effect of temperature change on an isolated corpuscle. In this case the temperature of an oil–saline bath was raised by passing current in an insulated nichrome wire, which circled around the bottom of the bath.

Ringer's solution. The mammalian Ringer's solution employed in the present experiment had the following composition (g/l.): NaCl 9·5, KCl 0·075, $CaCl_2$ 0·10 and $NaHCO_3$ 0·10.

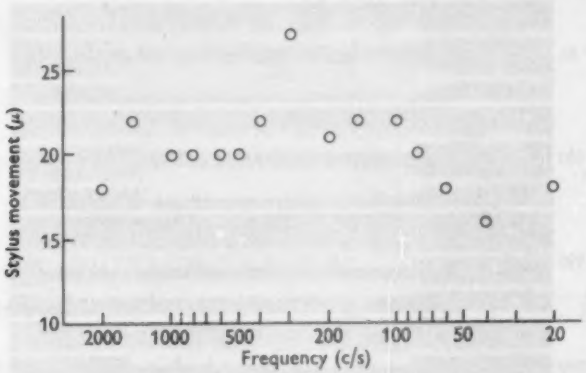

Fig. 1. Movement of the stylus attached to the piezo-electric bender element under sinusoidal voltages of varying frequency (see Text for calibration method). Applied voltages were 120V from peak to peak of alternating currents. Semi-log scale.

RESULTS

Frequency–threshold relationship in isolated corpuscles

The optimal frequency for stimulation of the corpuscle by sinusoidal vibration was found to be around 150–200 c/s at the room temperatures of 21–26° C. The threshold increased as the frequency was lowered below 150 c/s and at 10–20 c/s it was sometimes difficult to excite the corpuscle even with a voltage of 120 V applied to the crystal. The threshold also rose as the frequency was increased above 200 c/s. The pattern of impulses was such that at low frequencies the corpuscle responded to one cycle of the vibration with one or two impulses, but at frequencies of more than 200 c/s it responded to two or more cycles of the vibration with one impulse. To produce 1:1 relationship between the impulse and each cycle of the vibration the stimulus strength had to be increased with an increase in the vibration frequency. However, with a frequency of 500 c/s or more the threshold rose to such a degree that discharge from the corpuscle was not produced, even when the amplitude of the stimulus was increased to

about 50 times the threshold for a short rectangular mechanical pulse (Fig. 2).

The determination of a frequency–threshold curve was made in the following way: first, the threshold was measured for rectangular mechanical pulses of about 10 msec duration, which represents the rheobase (V_0); secondly, the threshold was determined for sinusoidal vibration of varying frequency (V, peak-to-peak value of the oscillation), and thirdly,

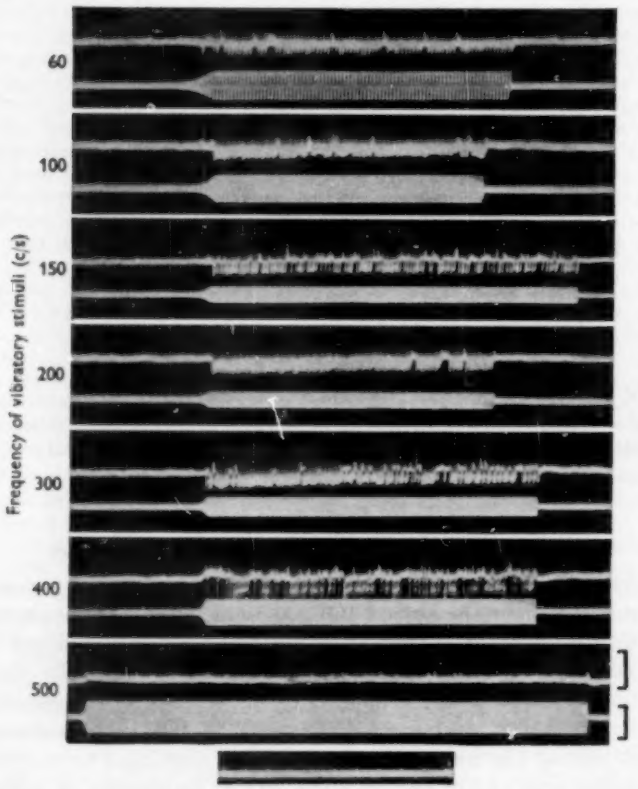

Fig. 2. The response of the isolated Pacinian corpuscle to sinusoidal vibration of various frequencies and of just threshold strength. In the upper trace impulses and receptor potential were recorded together. Receptor potentials and abortive spikes, possibly originated from the intracorpuscular node, can be seen when impulses are absent. The lower trace shows sinusoidal oscillatory voltages applied to the bender element. Calibration voltage for the impulse: 200 μV, and for the vibration: 40 V for top record and 20 V for other records. Room temperature, 25° C. Time marker 1 sec.

V_0 was measured again. The V/V_0 values were plotted against the vibration frequency. The threshold for vibration was taken as the stimulus strength at which the corpuscle showed steady discharge, but not necessarily a 1:1 response. If the threshold was determined by a 1:1 response, the frequency–threshold relation curve showed a much steeper rise when the frequency was increased from the optimum.

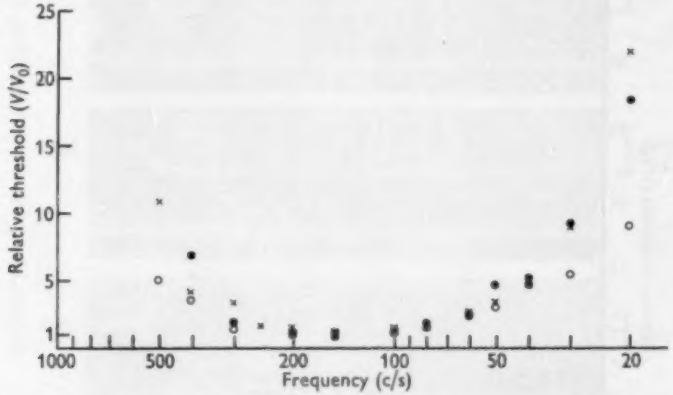

Fig. 3. The relation between vibration frequency and threshold of isolated Pacinian corpuscles for initiation of impulses. Three different sets of experiments are shown (×, ○, ●). × : Vibration was suddenly applied to the corpuscle (21° C). ○, ● : Slowly increasing vibration to finite amplitude was applied to the corpuscle (23 and 24° C). In this figure and in Figs. 4 and 6 ordinates are expressed by the ratio of the threshold for vibration to that for rectangular mechanical stimulus of 10 msec duration. Semi-log. scale.

In 10 out of 11 corpuscles examined at room temperature of 21–26° C the optimal frequency, i.e. the frequency with the lowest threshold for steady excitation of the corpuscle, was around 150–200 c/s, and the corpuscles followed only up to 400–500 c/s. In one exceptional case the corpuscle responded to vibration at 1000 c/s and its optimal frequency lay at a higher frequency than 200 c/s. Frequency–threshold curves of three corpuscles are shown in Fig. 3. At a frequency of 150–200 c/s the threshold was sometimes smaller than that for rectangular mechanical pulse (note in Fig. 3). At the low-frequency range the corpuscle usually followed vibrations of 10–20 c/s.

Experiments on undissected corpuscles with intact circulation

Several measurements of the frequency–threshold relationship were made on the Pacinian corpuscle *in situ*. Four examples from among seven experiments carried out are shown in Fig. 4, the remaining three giving

similar results. It will be seen in this figure that corpuscles followed the frequency of vibration up to 600–1000 c/s and that the threshold rose more steeply in the low-frequency range than it did in isolated corpuscles (Fig. 3), so that it was difficult to stimulate the corpuscle with a frequency of 20–30 c/s. By comparing Figs. 3 and 4 it can be seen that in the corpuscle *in situ* the V/V_0 value was 30 with the vibration frequency of 30–40 c/s,

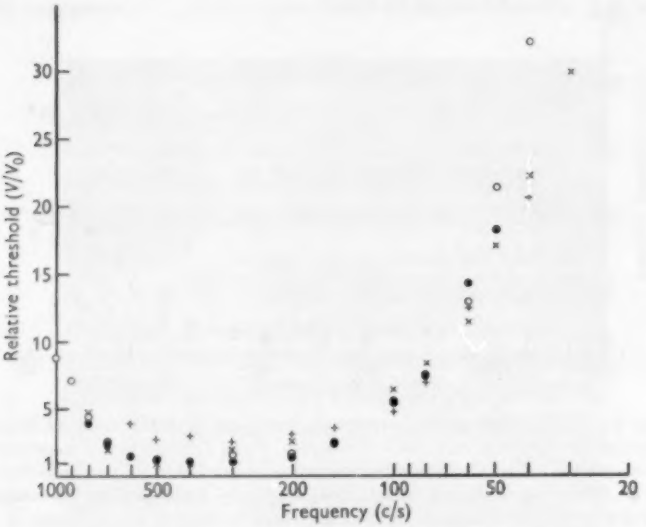

Fig. 4. The relation between vibration frequency and threshold of undissected corpuscles with intact blood supply. Four different sets of experiments are shown (×, +, ○, ●). ×, +: Vibration was suddenly applied. ○, ●: Slowly increasing vibration to finite amplitude was applied. Semi-log. scale.

while in the isolated corpuscle it was less than 10. In addition to this, in the undissected corpuscle the optimal frequency was near 300 c/s, compared with 150–200 c/s in the isolated corpuscle. In the high-frequency range the corpuscle with an intact circulation followed vibration at 800 c/s regularly in most cases, and still showed steady discharge at 1000 c/s (Fig. 5).

Effects of different temperatures on the response of the corpuscle

One successful experiment was performed in which the bath temperature was changed from 24 to 37° C. The frequency–threshold relationship was determined at both temperatures, and is shown in Fig. 6. At the higher temperature the corpuscle followed the vibration up to 1000 c/s, but the threshold rose more steeply in the low-frequency range than it did at the

lower temperature. Figure 6 also indicates that the discrepancy in the behaviour of the corpuscles when they are isolated and undissected is due to temperature difference, and not to the presence or absence of circulation.

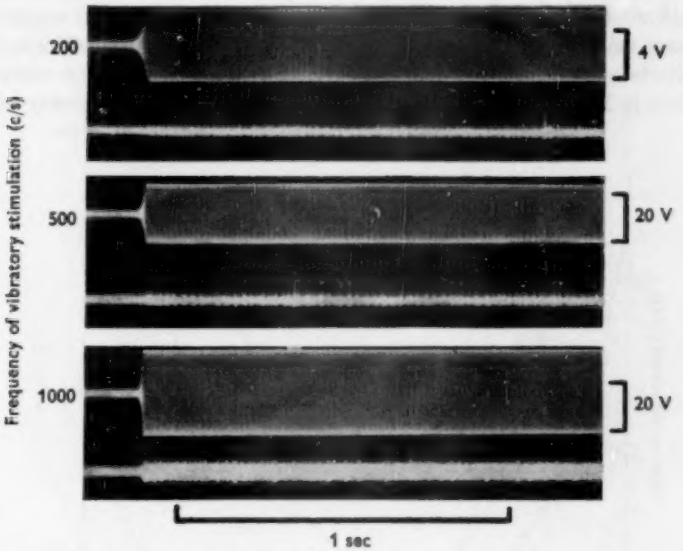

Fig. 5. The response of the undissected corpuscle to high-frequency vibration of just threshold. The base line of the lower trace is distorted because of interference of the stimulating voltage to the amplifier. Upper trace shows the voltage applied to the bender element. Numerals at the left indicate the frequency of vibratory stimulation (c/s).

In Fig. 6, as in Figs. 3 and 4, the threshold for vibration is expressed relative to that for a rectangular mechanical pulse. The latter, however, differed at the two temperatures, being 7·3 V at 24° C and 4·8 V at 37° C. In order to compare the absolute values of threshold for vibrations of varying frequency at these two temperatures, the former is plotted against the vibration frequency in Fig. 7. In this figure it can be seen that the absolute value of threshold for vibration at each optimal frequency hardly differs at the two temperatures, but that when the temperature is higher the threshold is lower for the frequency of more than 200 c/s and higher threshold is obtained below 200 c/s.

Receptor potential produced by sinusoidal vibration

The receptor potentials of isolated Pacinian corpuscles, produced by sinusoidal vibrations of various frequencies, were recorded at room tem-

peratures in order to obtain the quantitative relationship between the frequency of vibration and the amplitude of the receptor potential. As is seen in Fig. 8, the amplitude as well as the rate of rise of the receptor potential corresponding to each cycle of the vibration of the same intensity is smaller when the frequency is less than 100 c/s, and also the amplitude becomes smaller when the frequency is raised above 100–200 c/s. The amplitude of the receptor potential elicited by each cycle of the vibration is shown in Figs. 9 and 10 in relation to the frequency and intensity of the

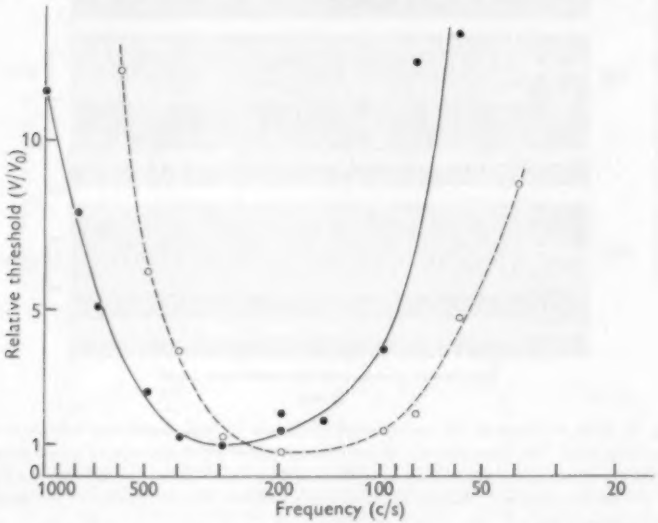

Fig. 6. Effect of temperature change on the frequency–threshold relationship of an isolated corpuscle. ○, 24° C; ●, 37° C. Semi-log. scale.

vibration. In Fig. 9 it is clear that the receptor potential becomes greater when the frequency is increased from 20 to 200 c/s, and then declines in amplitude with a further increase of the frequency. Comparing Fig. 9 with Fig. 3 it is evident that both the threshold–frequency relation and receptor-potential–frequency relationships have their optimal frequencies at 150–200 c/s. Thus the gradual rise in threshold for impulse initiation in the corpuscle on either lowering or raising the vibration frequency is mainly attributable to the reduction in the amplitude of the receptor potential.

A set of curves relating amplitude of receptor potential to stimulus strengths for rectangular pulse and sinusoidal movement are presented in Fig. 10. This is very similar to Fig. 11 of Gray & Sato (1953), and indicates

that the rate of rise of the stimulus determines the amplitude of the receptor potential. Although the rate of rise of each receptor potential for each cycle of vibration was not measured quantitatively, it is evidently reduced when the frequency becomes lower, as is seen in Fig. 8. This, together with the reduction in amplitude, might be responsible for the rise in threshold on lowering vibration frequency. In Fig. 9 the maximal

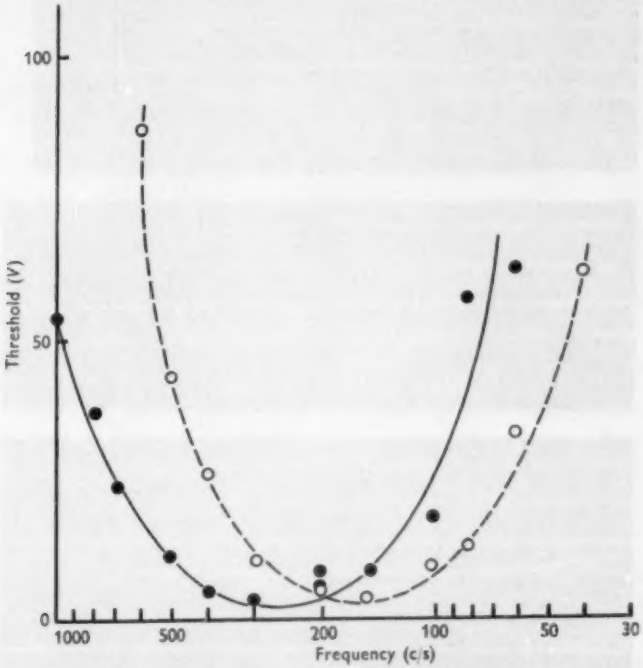

Fig. 7. Effect of temperature change on the absolute threshold of an isolated corpuscle for sinusoidal vibration of various frequencies. The same corpuscle as in Fig. 6. ○, 24° C; ●, 37° C. Semi-log. scale.

receptor potential to vibration of the same stimulus strength lies at 200 c/s when it is weak, but with an increase in the strength the frequency at which the maximal receptor potential is obtained tends to shift from 200 to 100 c/s. In two other similar experiments the maximal receptor potential was obtained by vibration at 100 c/s. There is, therefore, a slight difference regarding the optimal frequency in the results shown in Figs. 3 and 9, both of which were carried out at similar temperatures. The discrepancy might be explained by the possibility that the rate of rise of the receptor potential in response to the vibration of 200 c/s may be greater than that to 100 c/s, and this has

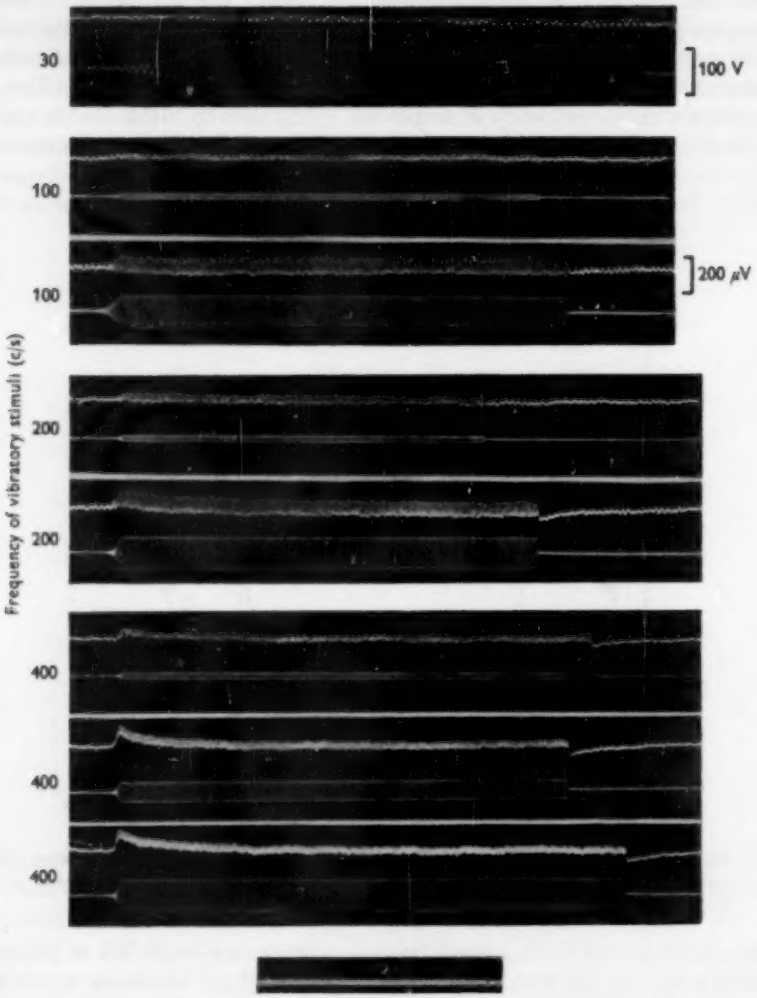

Fig. 8. The receptor potential in response to vibration of various frequencies and strengths. In each frame receptor potentials are shown in upper trace and the applied vibration in lower trace, the frequency being indicated at the left. The upper voltage calibration refers to the voltage applied to the bender element, the lower to the response. Procanized corpuscle at room temperature (26° C). Time marker 1 sec.

a greater influence for initiating the impulse from the first node of Ranvier than merely the amplitude of the receptor potential.

In Fig. 8 the receptor potentials show a summation to vibration of more than 150 c/s, attaining a maximum depolarization and then gradually falling to a steady level. This figure also shows that there is a slow development of a hyperpolarization to a steady value; the hyperpolarization appeared to outlast the stimulus for some time, but its accurate time course was hard to determine because of the amplifier time constant. No summation of the receptor potentials or building up of the hyperpolarization was observed below the frequency of 100 c/s. Figure 2 illustrates both

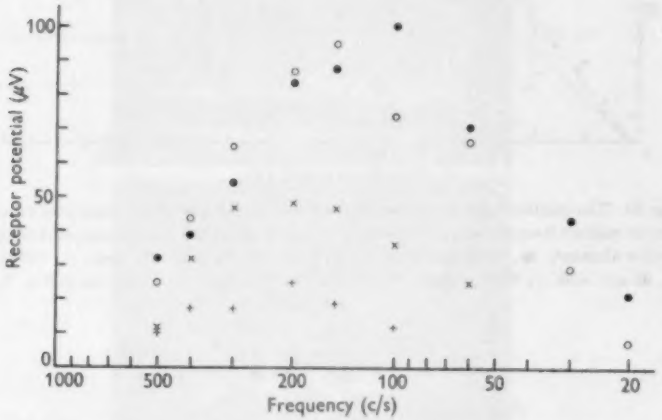

Fig. 9. The relation between receptor potential amplitude and vibration frequency at various intensities. The voltages applied to the bender element are (V): 119, ●; 44·5, ○; 30·5, ×; and 7, +. Procanized corpuscle at room temperature. Semi-log. scale.

the receptor potential and the impulse initiated therefrom, when the stimulus near threshold strength was applied to the corpuscle. Two facts are worth mentioning here. First, there were abortive impulses when impulses failed to discharge: these abortive impulses are possibly initiated from the first node of Ranvier inside the corpuscle (Quilliam & Sato, 1955; Diamond, Gray & Sato, 1956). Secondly, impulses were not elicited at a frequency of 500 c/s, although the summed receptor potential was greater than the firing level at which impulses were initiated with vibrations below 100 c/s. In Fig. 11 the magnitude of the summed receptor potential is shown in relation to the frequency and strength of vibration. It increases with an increase in frequency up to 400 c/s, and it declines sharply with a further increase in frequency. Apparently the level of the summed receptor

potential is not directly related to the impulse initiation, but rather each receptor potential for each cycle of the vibration seems to be responsible for the initiation of the impulse. Evidently the steady depolarization of the receptor membrane in the corpuscle cannot itself initiate the impulse at the first node of Ranvier.

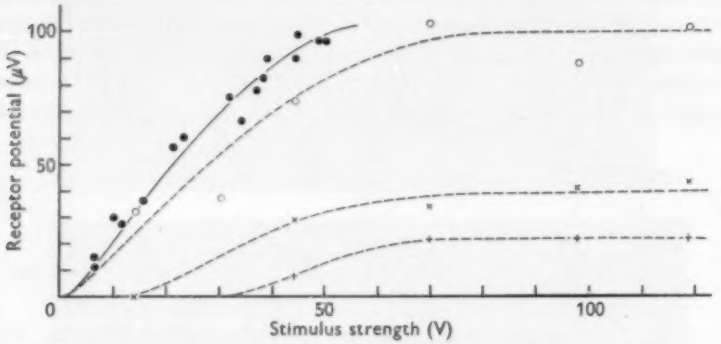

Fig. 10. The relation between receptor potential amplitude and intensity of vibration at various frequencies. Intensities are expressed as the voltages applied to the bender element. ●, rectangular mechanical pulses, duration 20 msec; ○, 100 c/s; +, 20 c/s; and ×, 300 c/s sinusoidal vibration. The same corpuscle as in Fig. 9.

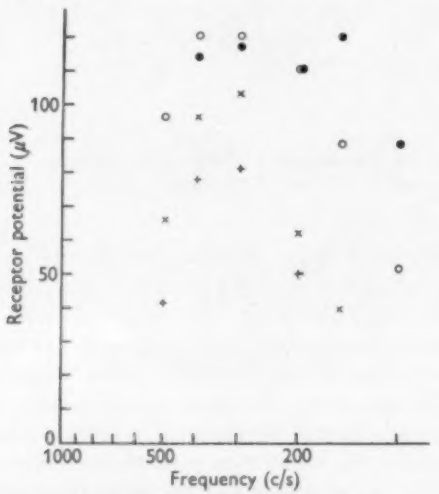

Fig. 11. The peak amplitude of the summed receptor potentials produced by vibrations of various frequencies and intensities. Voltages applied to the bender element are (V): 119, ●; 44·5, ○; 30·5, ×; and 15, +. The same corpuscle as in Fig. 9. Semi-log. scale.

Repetitive discharge from Pacinian corpuscles

During the course of these experiments the corpuscle often responded to short mechanical pulses with repetitive discharge, which sometimes lasted for as long as 50 msec (Fig. 12). The duration of this repetitive discharge depended on the stimulus strength, but not on the duration of the stimulus.

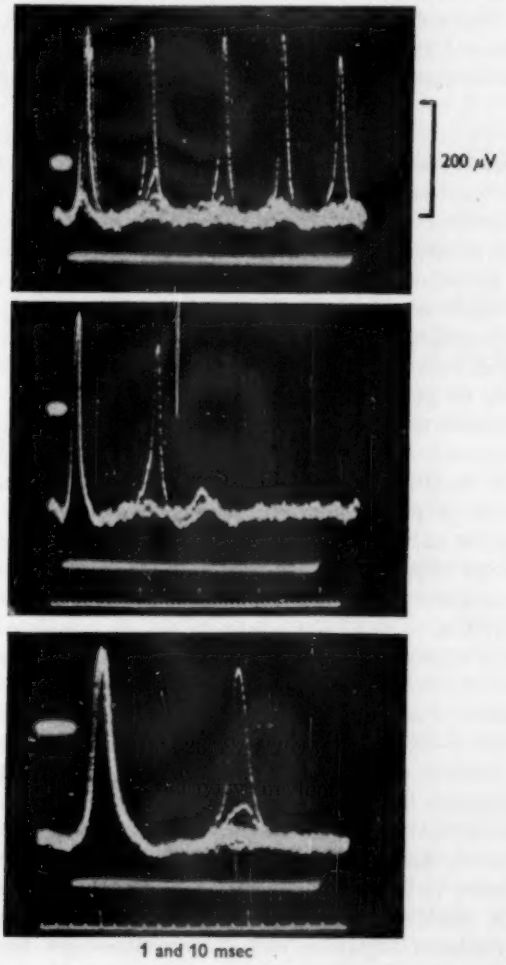

Fig. 12. Repetitive impulses from the corpuscle produced by a short mechanical stimulus of 2 msec duration. Several sweeps were superimposed in each frame.

When the receptor potential and the impulse were recorded together, it was easily found that there was a series of small receptor potentials, spaced with an interval of 5–10 msec, in response to short mechanical pulses, and that an impulse was sometimes initiated from each receptor potential. A typical example is shown in Fig. 12, where small spikes of all-or-none nature are observed on the superimposed traces when the impulse failed to discharge. It seems clear from these observations that the receptor potential of the corpuscle, when it produces repetitive discharge, is not a sustained potential change, but rather an oscillatory one; the interval of impulses is determined by that of the oscillatory potential.

DISCUSSION

The present experiments reveal that the range of frequency response of Pacinian corpuscles in the mesentery of the cat to sinusoidal vibration is from 10 to 500 c/s at 21–26° C and from 20 to 1000 c/s at about 37° C, and that there is an optimal frequency at about 150 and 300 c/s, respectively. From the experiments described the difference in both the range of frequency response and the optimal frequency at different temperatures can be attributed to the difference in the temperature, while the presence or absence of a circulation makes no significant difference to these properties of the corpuscle.

The increase in the threshold for vibration at low-frequency range in the Pacinian corpuscle may be due to the reduction in the amplitude and rate of rise of the receptor potential, and the latter is accounted for by the fact that both the amplitude and the rate of rise of the receptor potential become smaller as the rise time of each cycle of the vibratory stimulus becomes larger with a decrease in the frequency (Gray & Sato, 1953). This would also account for the lack of response below a certain frequency, by which the critical slope of the corpuscle can be calculated. The approximate critical slopes, calculated from Figs. 3 and 4, are 20 rheobases/50 msec = 0·4 rheobases/msec for the isolated corpuscle at 21–26° C and 30 rheobases/33·3 msec = 0·9 rheobases/msec for the corpuscle *in situ*. These are of the same order as 1·2 rheobases/msec obtained by Gray & Matthews (1951*b*), although my values are slightly less than theirs.

It is well known that the nerve fibre has an optimal frequency when it is excited by alternating currents of various frequencies. When the frequency of an alternating current is lowered below the optimum, the threshold rises, which is known to be due to accommodation of the fibre (Hill, 1936; Sato & Usiyama, 1950). If one compares both kinds of frequency–threshold curves, of Pacinian corpuscles and of nerve fibres, one finds that the rise in threshold produced by lowering the frequency is much steeper in the corpuscle than in the nerve fibre, indicating that the former has more rapid

adaptation than the latter. This is consistent with the experiments of Gray & Matthews (1951*b*), where they compared the adaptation of the corpuscle to the mechanical stimuli with the accommodation of its own axon to the electric currents and found that the former has a larger critical slope than the latter.

The rise in the threshold for vibration with frequencies above 200 c/s could be accounted for by the decrease in both the amplitude and the rate of rise of the receptor potential, even though the tendency for receptor potentials to sum will, presumably, have the opposite effect. Since the receptor potential following the preceding one shows depression when the interval between two in succession is less than about 10 msec (Gray & Sato, 1953; Diamond, Gray & Inman, 1958), the decrease in the amplitude and rate of rise of the receptor potential with frequencies above 200 c/s can be explained in terms of depression. The inability of the corpuscle to initiate an impulse with a vibration of high frequency such as 500 c/s or more might be accounted for by the possibility that the amplitude and rate of rise of the receptor potential for each cycle of vibration become too small to elicit impulses at the first node of Ranvier, or by the possibility that the node becomes inactivated by steady depolarization of the non-myelinated ending due to summed receptor potentials. The latter possibility can be tested by applying antidromic stimulation to the corpuscle during mechanical stimulation of about 500 c/s, and should be investigated with future experiments.

Recently Inman & Peruzzi (1961) have examined the effects of temperature on the corpuscle and have obtained a larger amplitude and rate of rise of the receptor potential by raising the temperature, with a Q_{10} of about 2 and 2·4, respectively. This is consistent with the present experiments, where the threshold for a rectangular mechanical pulse is lowered when the temperature is raised. Lowering of the threshold for vibration above the optimal frequency is, therefore, explained by an increase in the amplitude and rate of rise of the receptor potential and also probably by lesser depression at higher temperature. The steeper rise in the threshold for vibration below the optimal frequency at higher temperature indicates that the corpuscle adapts more rapidly to the mechanical stimulus at higher temperature.

The repetitive discharge of impulses in response to a short mechanical stimulus observed during the course of the present experiments is consistent with earlier observations by Scott (1949) and Alvarez-Buylla & Remolina (1959). The fact that the interval of repetitive impulses is always regular and of the order of 5–10 msec would exclude the possibility that this repetitive discharge might be due to the vibration transmitted from the outside, and also it cannot be attributed to oscillation of the stylus

due to insufficient damping, because the damping frequency of the stylus is more than 1000 c/s. It is also evident that this repetitive discharge observed in the present experiments is of a different nature from that found by Inman & Peruzzi (1961), because (i) the repetitive discharge lasted sometimes for about 50 msec (Fig. 12, top record), which is far longer than the duration of the receptor potential, and (ii) small oscillations of the base line or receptor potentials can be seen when impulses failed to discharge.

This point is made clearer by Fig. 13, which consists of unpublished photographs by Sato & Ozeki. In *A* a mechanical stimulus of just threshold was applied to the corpuscle, and in *B–E* the corpuscle–nerve–fibre preparation was procanized successively, keeping the stimulus strength constant. Procaine was applied to the central pool first between *B* and *C*, and then the corpuscle was procanized between *D* and *E* in order to abolish the impulse completely. It will be clear from this figure that the second impulse is not initiated at the falling phase of the receptor potential, but produced at the peak of the second receptor potential (see *A* and also compare *D* with *E*). This figure supports another observation, that the repetitive discharge was not produced by inadequate damping of the stylus. If the second impulse in *A–D* were produced by insufficient damping, one would observe the second receptor potential, in *E*, at the point where the second impulse had been initiated. One of the reasons why the Pacinian corpuscle in the present experiments showed repetitive discharges relatively often in response to a short mechanical stimulus is, presumably, that the mammalian Ringer's solution employed in the present experiments contains a relatively small amount of potassium chloride, compared with that used by other investigators.

From the results of the present experiments it has become clear that the range of frequency response of Pacinian corpuscles in the mesentery to sinusoidal vibration is the same as that of vibration receptors in the interosseous membrane and in the tibial periosteum of the hind limbs of cats (Hunt & McIntyre, 1960; Hunt, 1961). Since there is no anatomical difference between Pacinian corpuscles in different parts of the body, it is not surprising that they show similar properties to mechanical stimulation.

Human vibratory sensation has been extensively studied by Geldard and others (see Geldard, 1953). Geldard's measurements of the threshold for human vibratory sensibility show that the optimal frequency lies in the neighbourhood of 250 c/s and the frequency range in which the stimulus is perceived as a vibration is from 20 to 20,000 c/s, although it is very doubtful whether one can perceive the vibration at 20,000 c/s. At any rate, the frequency range and the optimal frequency of the human vibratory sensation are quite similar to those obtained with Pacinian corpuscles in the present experiments.

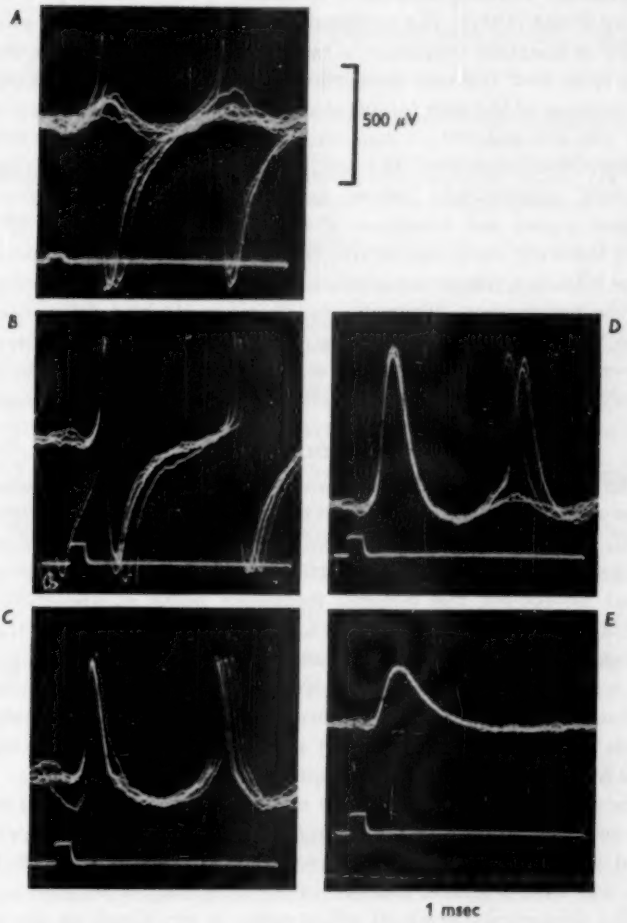

Fig. 13. Photographs showing repetitive impulses from the corpuscle. Several mechanical stimuli of 1 msec duration were applied to the corpuscle with a rate of 20 c/s and the responses were superimposed. Stimulus strength is just threshold in *A*, and is about three times the threshold and kept constant from *B* to *E*. Between *B* and *C* the proximal pool was procanized, and between *D* and *E* the peripheral pool was also procanized so that only the receptor potential remains in *E*. Unpublished photographs by Sato & Ozeki.

The effect on vibratory sensibility of changing the skin temperature was studied by Weitz (1941). His results show that the threshold for vibratory sensibility of a certain frequency is raised by cooling the skin, lowered by warming it up to 4° C above the normal temperature and raised again by a further increase of the skin temperature. He studied the threshold change for only 100, 256 and 900 c/s, and made no investigations on the vibration sensibility of low frequency. His results are to some extent consistent with the present experiments, where the threshold for both rectangular mechanical pulses and vibration of a frequency higher than 200 c/s is raised by lowering the temperature. However, in the present experiments, when the vibration frequency is below 200 c/s, the threshold is increased by raising the temperature. There seems, therefore, to be a difference in the response to low-frequency vibration between the results of the present experiments and those of Weitz. It would be interesting to see what kind of sensory receptors are concerned with the human vibratory sensation.

SUMMARY

1. The response of isolated and undissected Pacinian corpuscles in the mesentery of the cat to sinusoidal vibrations of various frequencies was recorded, and the frequency–threshold relationship was determined.

2. At room temperatures of 21–26° C the corpuscle followed vibration of about 20–400 c/s, the optimal frequency being at 150–200 c/s. The threshold became higher with either an increase or a decrease of the vibration frequency from this optimum, and in almost all cases no impulse discharge was initiated from the corpuscle in response to the vibration of more than 500 c/s. At a temperature of 37° C, or with the undissected corpuscle with intact circulation, it responded persistently to vibration from 40 to 1000 c/s, the optimal frequency lying around 300 c/s.

3. The receptor potential from the non-myelinated ending was recorded in response to the vibration. Its magnitude to each cycle of the vibration attained its maximum when the frequency was raised from 20 to 150–200 c/s, and with a further increase in the frequency it declined again.

4. The frequency–threshold relationship is explained on the basis of receptor potential behaviour.

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ANION PERMEABILITY OF THE SYNAPTIC AND NON-SYNAPTIC MOTONEURONE MEMBRANE

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It was discovered by Coombs, Eccles & Fatt (1955*a*) that the hyperpolarization of the inhibitory post-synaptic potential (IPSP) changed to a depolarization when Cl^- ions were injected either by diffusion or electrophoretically into cat spinal motoneurones. This effect was satisfactorily explained by assuming that, when activated, the inhibitory post-synaptic membrane is permeable to Cl^- ions. A similar effect was obtained for Br^-, NO_3^- and SCN^- ions, but not for HCO_3^-, $CH_3CO_2^-$, SO_4^{2-}, HPO_4^{2-} and glutamate ions. Since the ions in the former group are smaller in their hydrated form than those in the latter, it was postulated that in the activated state the inhibitory post-synaptic membrane has a sieve-like structure with a pore size large enough to allow the passage of SCN^- ions, yet small enough to block bicarbonate and acetate ions. A differential permeability of the inhibitory post-junctional membrane was also reported on crustacean muscle (Boistel & Fatt, 1958) and crustacean stretch receptor cell (Hagiwara, Kusano & Saito, 1960) for Cl^- ions on the one hand, there being impermeability to sulphate and large organic ions on the other. The first aim of the present investigation was to employ a more extensive anion series in order to test the postulate that the differential permeability is explicable by the pore size. Eleven anion species have now been established as penetrating and another sixteen as non-penetrating through the activated inhibitory post-synaptic membrane of cat spinal motoneurones. A preliminary report of some of these findings has been given (Araki, Ito & Oscarsson, 1961).

Coombs *et al.* (1955*a*) observed that the size of the IPSP, changed by injection of Cl^-, Br^-, NO_3^- and SCN^- ions, recovered exponentially with a time constant of about 30 sec. In the course of the present experiments it was found that the time course of the IPSP recovery varied according to the injected anion species. Since the recovery is caused by the removal

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of injected anions from the cell, its time constant should be related to the permeability of the cell membrane in general. Further investigation of these time constants has been the second aim of the present experiments.

METHODS

The experiments were performed on 25 cats under pentobarbitone sodium anaesthesia. The cord was transected at the L2 level. The nerves from posterior biceps-semitendinosus (PBST), gastrocnemius-soleus, plantaris, flexor digitorum plus flexor hallucis longus, and peroneal muscles were dissected and mounted on stimulating electrodes in a paraffin pool. The quadriceps nerve was stimulated by a buried electrode. The technique of intracellular recording was the same as that described by Eccles, Fatt, Landgren & Winsbury (1954). PBST motoneurones were particularly searched for, because they receive a large IPSP from Ia afferents in the quadriceps nerve (Brock, Coombs & Eccles, 1952). The motoneurones were identified by antidromic stimulation of the cut L7 and S1 ventral roots and by the synaptic potentials evoked by Group Ia volleys in various nerves (Eccles, Eccles & Lundberg, 1957*a*). A pure IPSP could be obtained by using a stimulus strength subthreshold for Ib fibres, which was particularly easy with the quadriceps nerve on account of the frequency of a good threshold separation of the Ia and Ib fibres (Bradley & Eccles, 1953). The afferent volleys were monitored with a surface electrode placed at the upper L7 dorsal root entry. The IPSP produced by the Renshaw cell pathway was occasionally used (cf. Coombs *et al.* 1955*a*) and was obtained by the ventral root stimulation just subthreshold for the axon belonging to the impaled motoneurone, or by a maximal ventral root stimulus when the antidromic invasion was blocked (Eccles, Fatt & Koketsu, 1954). In some cases polysynaptic IPSPs evoked in extensor motoneurones by Group Ib or II volleys, or by a volley from the peroneal nerve, containing cutaneous afferents (cf. Eccles, Eccles & Lundberg, 1957*a*, *b*; Eccles & Lundberg, 1959) were employed for qualitative observations of anion effects (Table 2).

Capillary glass electrodes were boiled in distilled water under reduced air pressure at about 60 °C. The water in the shaft of the electrode was then sucked out through a thin vinyl tube and substituted by the solutions listed in Table 1, which contain various anions as either potassium or sodium salts. Potassium salts were preferred, unless their solubility was very small as was the case with ClO_4^-, ClO_2^- and BrO_3^-, but not necessarily so because the effect of sodium ions upon the IPSP by diffusion from the electrode tip was always negligible. More than 24 hr after the filling with solutions micro-electrodes with resistance of 8–20 MΩ were selected; but with solutions containing relatively low concentrations of $KMnO_4$ or $Na_2C_2O_4$ resistances up to 50 MΩ were tolerated. Solutions of NaF, or $NaMnO_4$, or NaCN usually caused a blunting of the electrode tip, but among many electrodes prepared some remained with a tip diameter less than 1 μ and could be used successfully for impalement of motoneurones.

The micro-electrode was connected to a current generator through a 100 MΩ resistor, and anions were injected from the tip into the cell by passing currents of $3-10\times10^{-9}$A for 30–120 sec (cf. Coombs *et al.* 1955 *a*). The potentials were recorded through the same electrode that served for the injection. After application of the current there was usually a transient change of the tip potential of the electrode, amounting to 5–20 mV or more, either positive or negative, which diminished in most cases within 20–30 sec. This artifact potential was obtained in isolation by recording with the tip in the extracellular medium and by applying the same current that was used for injection. The intracellular resting potential was then derived by subtracting the artifact potential from the potential recorded inside the cell. This method of artifact compensation is not entirely satisfactory, as the shift of the electrode tip from the intracellular to the extracellular medium may cause a change in the artifact

potential, either for mechanical reasons or because of the different ionic composition of the two phases of the media. However, it was useful in showing how far the recorded potential was reliable in indicating the resting potential of the cell. Electrodes with large artifact potentials were rejected.

TABLE 1. Solutions used for filling micro-electrodes

Substance	Concentration (M)	Substance	Concentration (M)
KCl	3	NaN_3	5
KBr	4	$NaClO_2$	5
KI	3	$NaClO_4$	1, 3 and 5
KNO_2	3	$NaBF_4$	3
KNO_3	5	$NaHCO_2$	5
KSCN	5	$NaBrO_3$	2
$KHCO_3$	2	$NaHSO_3$	3·4
$KCH_3CH_3CO_3$	3	$Na_2C_2O_4$	0·3
$K_3Fe(CN)_6$	0·8	NaF	0·8
$K_4Fe(CN)_6$	0·8	$NaCH_3(CH_3)_2CO_3$	5
K-Citrate	2	Na-L-Glutamate	5*
$KMnO_4$	0·4	NaCN	3
$NaMnO_4$	3		

* pH was adjusted to 8.

Any potential appearing between the silver wire dipped in the solution of the micro-electrode and the indifferent electrode in contact with the back muscles of the cat was cancelled by an electromotive force inserted between the indifferent electrode and the earth, so as to prevent currents driving ions from the electrode. The current passed through the micro-electrode for injections was monitored by a differential d.c. recording of the potential drop which was set up across 100 MΩ resistor by the current. The effective resistance of the 100 MΩ resistor, as the input impedance, was raised to about 5000 MΩ by a series feedback device (Ito, 1960). The resting and action potentials were recorded through a cathode follower by a d.c. amplifier. The resting potential was registered by an ink-writing recorder while the action potential and the synaptic potentials, the latter being amplified by a condenser-coupled amplifier (time constant usually 20 msec, but 500 msec when recording Renshaw IPSP), were displayed by a cathode-ray oscilloscope. The changes of the synaptic potentials and of the action potential following the injection of anions were recorded by photographing sweeps repeated at a frequency of 5/sec. The photographic film was automatically advanced every 4 sec, giving records with about 20 superposed traces.

RESULTS

Penetrating anions

The injection of NO_2^-, I^-, BF_4^-, ClO_3^- and HCO_2^- ions by a hyperpolarizing current of $3-10\times10^{-9}$A for 30–120 sec readily induced a reversal of the IPSP. For example, Fig. 1 illustrates the reversal and recovery of the Ia IPSP(A) and of the Renshaw IPSP(B) by the injection of NO_2^- ions. As pointed out by Coombs *et al.* (1955*a*), the reversed IPSP is not a mirror image of the original hyperpolarization; it attains its peak earlier and decays more quickly than the latter (Fig. 1). When the reversed IPSP turns back to a hyperpolarization in the course of recovery, there is usually a diphasic stage; a small depolarization being followed by a hyperpolarization (Fig. 1A, at 88–92 sec), as already reported and explained by

Coombs *et al.* (1955*a*). The earlier summit of the reversed IPSP is particularly noticeable for the Renshaw IPSP (Fig. 1B, at 52–56 sec). The change in the size of the IPSP by injection of NO_2^- ions in another PBST motoneurone is plotted in Fig. 3A. Because of the above-mentioned change in

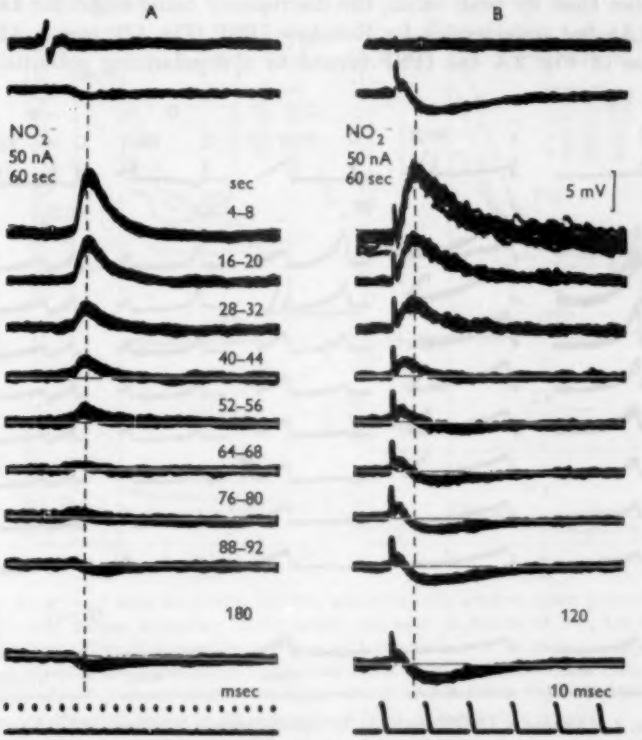

Fig. 1. Effects of electrophoretic injection of NO_2^- ions into motoneurones. A, Ia IPSP in a PBST motoneurone evoked by quadriceps Ia volley. B, Renshaw IPSP in a motoneurone, the innervation of which was not identified, induced by a maximal L7 ventral root stimulation. Records in the top row show incoming volleys recorded from the surface of the cord (upper trace) and the control IPSPs thereby evoked before the injection (lower traces). Records from the second to the ninth row illustrate IPSPs at the indicated time (identical in A and B) after the injection of NO_2^- ions by the passage of a current of 5×10^{-8}A for 60 sec. The bottom records are IPSPs at the end of recovery, its approximate time being shown in records. In this and in successive figures IPSPs are illustrated with positivity upwards. Reference base lines are drawn in traces at the fifth to tenth row, and vertical interrupted lines are through peaks of reversed IPSPs, indicating the points in the time course of IPSPs where their sizes are measured (see text). Note different time scales for A and B. All records were formed by the superposition of about twenty faint traces.

the time course, the size of the IPSP was not measured by its peak value, but by the amplitude at a fixed time after its beginning, corresponding to the peak of the largest depolarizing IPSP, as shown by the interrupted line in Fig. 1. Hence the plotted size of the hyperpolarizing IPSPs in Figs. 3–6 is smaller than its peak value, the discrepancy being slight for Ia IPSP (Fig. 1A), but considerable for Renshaw IPSP (Fig. 1B) (see p. 424). In the case of Fig. 3A the IPSP turned to a depolarizing potential spon-

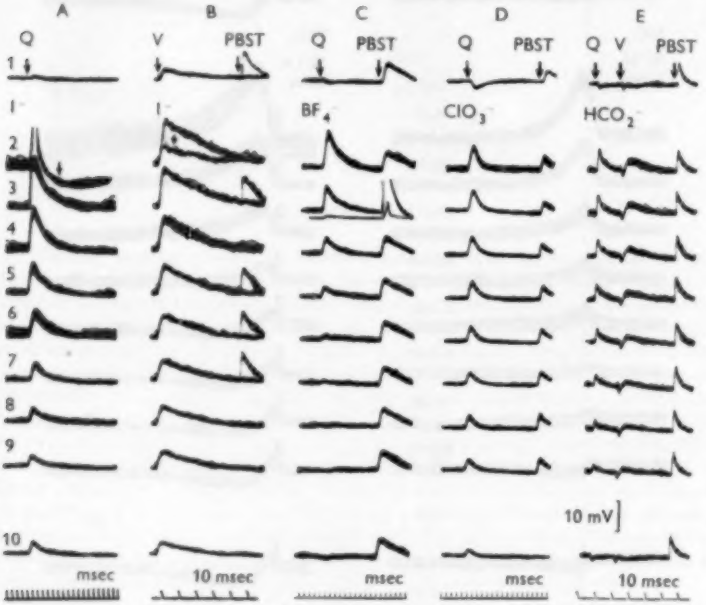

Fig. 2. Changes in IPSPs produced by electrophoretic injections of I^- (A and B), BF_4^- (C), ClO_3^- (D) and HCO_3^- (E) ions. Ia IPSPs (A, C, D and E) and Renshaw IPSPs (B and E) were obtained by quadriceps volleys (Q) and by L7 ventral root stimuli (V), respectively, in PBST motoneurones. Records in the first row are controls before the injections. A current of 5×10^{-8}A for 60 sec produced the injection. The time at which successive records from the second to the ninth row were taken was 0–4, 20–24, 40–44, 80–84, 100–104, 120–124, 140–144 and 160–164 sec in A, and the same as in Fig. 1 for others. Bottom traces are IPSPs at 180 sec in A, B and C, at 160 sec in D and at 360 sec in E after injections. In records at the second row of A and B the reversed IPSPs elicited spike potentials and traces of their after-hyperpolarization are marked by arrows. Monosynaptic EPSPs produced by volleys in the posterior biceps-semitendinosus nerve (PBST) are displayed at the end of sweeps by continuous (C, D and E) or intermittent (B) stimulation. In the third row of C a spike potential was obtained by an antidromic stimulation in place of the EPSPs, being shown in an extra trace with a gain of about one tenth of that for other records. Note two kinds of time scales for A, C, D and B, E.

taneously after impalement of the cell, apparently due to the NO_2^- ions that diffused out of the electrode (cf. Coombs *et al.* 1955*a*). The final value of the IPSP after the recovery is usually slightly different from the one before the injection; it remained often as a depolarizing potential (see Figs. 3A; 2A, B, D).

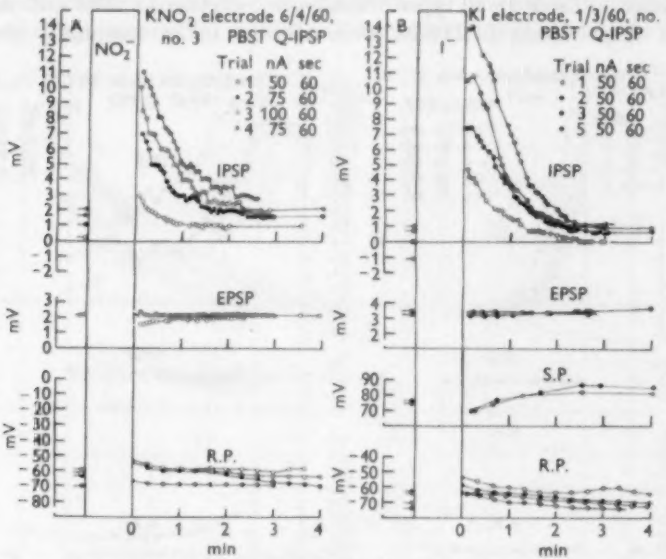

Fig. 3. Plot of sizes of IPSPs, EPSPs, antidromically evoked spike potentials (S.P.) and resting potentials (R.P.) before and after injections of NO_2^-(A) and I^-(B) ions. Plots of four trials into one and the same cell are superposed, the order of which are shown in the figure with the strength and duration of currents employed for injections. In A of this figure as well as in B of Fig. 4 spike potentials were not obtained, owing to the spontaneous block of antidromic invasion.

Changes in Ia IPSP and Renshaw IPSP by injection of I^- ions are shown in Fig. 2. Just after the injection (2nd row in Fig. 2A, B), the depolarizing IPSPs reached the critical level for generation of spikes, which was often observed after injections of the effective ions (cf. Coombs *et al.* 1955*a*). The time course of the IPSP recovery after four injections of I^- ions is shown in Fig. 3B. In Fig. 3 as well as in Figs. 4 and 5 the reproducibility of the effect of injections of any particular anion may be appreciated by the superposed plots of four successive trials into one and the same cell. Effects of BF_4^-, ClO_3^- and HCO_2^- ions upon the IPSP are qualitatively the same as NO_2^- and I^- ions, as seen in Fig. 2C, D, E, but the time course of the IPSP recovery is particularly slow for ClO_3^- and HCO_2^- ions (see Figs. 4B and 5A and Table 4).

The effect of injections of the above anions was easily reproducible and similar to the effect of the previously tested Cl^-, Br^-, NO_3^- and SCN^- ions. The latter ions have been re-tested in the present experiments, and the number of trials of injections is listed in Table 2. As observed by Coombs *et al.* (1955*a*) and also as revealed by some of the anions in the present experiments (Table 2), all types of inhibition, whether Ia IPSPs, Renshaw IPSPs or polysynaptic IPSPs, were affected in an essentially similar

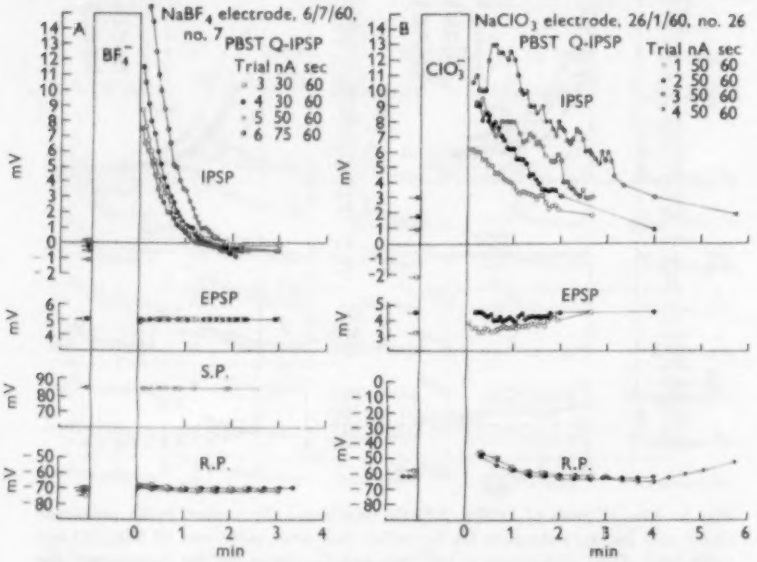

Fig. 4. Plot of intracellular potentials before and after injections of BF_4^- (A) and ClO_3^- (B) ions. Conventions and symbols as for Fig. 3.

manner. The change of the IPSP size caused by the injection of these anions is given in Table 3 by the algebraic difference between the IPSPs before and just after the injection. The figures in the five PBST motoneurones of this table are the largest among cells examined with each anion (Table 2). It appears in Table 3 that the change of the IPSP is particularly large with NO_3^- and SCN^- ions, while it is relatively small with ClO_3^- and HCO_2^-. However, since the IPSP size, or strictly speaking the IPSP conductance (cf. Coombs *et al.* 1955*a*), depends on the density of inhibitory innervation, and hence varies considerably in the population of PBST cells, it is not possible to compare the effect of injections simply by the IPSP size. The values in Table 3 and comparison of Figs. 3, 4 and 5 suggest that no quantitative difference has been shown to exist between the effects of these various anions in reversing the IPSP.

N_3^- ions are also effective in converting the IPSP to a depolarizing potential, but, since the injection of this ion species causes a reduction of the membrane potential, the pattern of changes of the IPSP is somewhat different from those observed with the anions of Table 3. Even injections of the anions of Table 3 often caused damage to the cell, presumably owing to the mechanical shift of the electrode tip, relative to the cell, that was caused by the swelling of the cell during the injection (see p. 424).

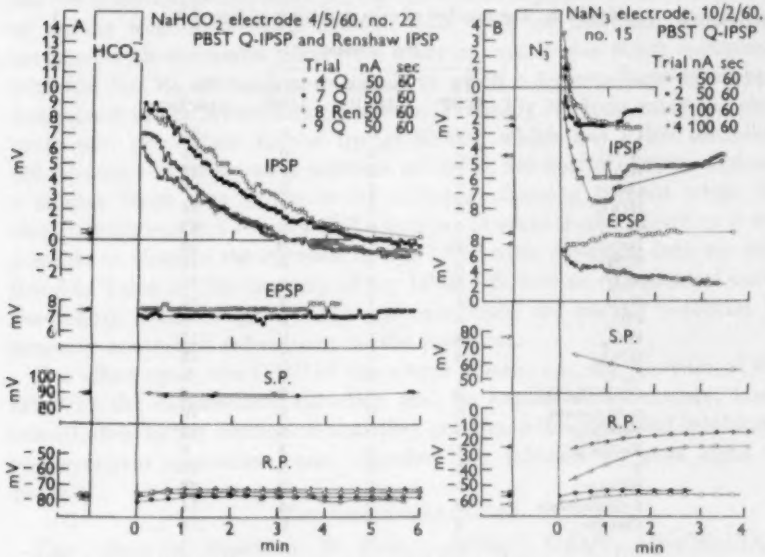

Fig. 5. Plot as in Fig. 3 of intracellular potentials for injections of HCO_3^- (A) and N_3^- (B) ions. Note that in trial 8 of A the size of Renshaw IPSP is plotted in place of Ia IPSPs in other trials.

When the cell was in this way destroyed or damaged irreversibly, these results were not included in Table 2. With N_3^-, however, the damage of the cell seemed to be induced by the ion, owing possibly to its well known effect as a metabolic inhibitor (cf. Ling & Gerard, 1949; Gerard & Doty, 1950). For example, in the experiments of Fig. 5B, the first two injections by 5×10^{-8} A affected the membrane potential little, while the following two trials with larger amounts of injected ions caused a remarkable reduction of the membrane potential. Since a depolarizing IPSP is depressed or even reversed by reduction of the resting potential (Coombs *et al.* 1955*a*), the induced change in the IPSP might have been much larger in the later two trials than in the first two, if there had been no reduction of the membrane potential. This reduction of the membrane potential was usually pro-

gressive, and correspondingly the reversed IPSP was converted rapidly to a hyperpolarizing potential and was later reduced again, as is seen in Fig. 5B. There were 51 injections into 20 cells, in all of which a depolarization of the membrane was observed to some degree. However, when a large reduction of the membrane potential was avoided by a relatively small dose of injected ions, the small change so induced in the IPSP recovered in an approximately exponential way. Hence it is concluded

TABLE 2. The number of cells and trials of injections

Ion	No. of cells: Ia IPSP	Renshaw IPSP	Polysyn. IPSP	No. of injections
I^-	8	0	0	25
NO_3^-	5	3	0	21
BF_4^-	7	0	1	27
ClO_3^-	7	2	0	19
HCO_2^-	13	1	2	38
Cl^-	5	0	0	13
Br^-	6	1	0	16
SCN^-	5	1	0	18
NO_2^-	8	1	0	21
N_3^-	11	7	2	51
ClO_4^-	6	0	0	8
BrO_3^-	8	1	0	17
HSO_3^-	6	0	2	13
$C_2O_4^{2-}$	1	0	2	7
$CH_3CH_2CO_2^-$	3	0	2	8
n-Butyrate	0	0	6	10
$Fe(CN)_6^{3-}$	5	0	2	13
$Fe(CN)_6^{4-}$	1	0	3	4
F^-	4	0	9	15
HCO_3^-	5	1	0	23
L-Glutamate	0	2	4	14
Citrate	3	0	0	4

TABLE 3. Changes of the IPSP by anion injection (mV)

Ion	Motoneurones: *a*	*b*	*c*	*d*	*e*
Cl^-	9·0 (1)	7·9 (3)	7·4 (3)	6·8 (2)	4·8 (1)
Br^-	11·6 (3)	11·1 (4)	7·5 (1)	7·0 (2)	6·5 (2)
I^-	11·2 (3)	11·0 (3)	8·2 (4)	7·8 (4)	7·2 (1)
NO_2^-	14·4 (3)*	12·8 (4)	10·0 (1)	4·4 (1)	4·0 (1)
NO_3^-	10·1 (2)	8·8 (1)	7·4 (2)	7·2 (2)	7·1 (4)
SCN^-	14·8 (5)	12·8 (3)	11·8 (3)	10·1 (2)	8·4 (2)
BF_4^-	11·9 (6)	8·4 (5)	7·9 (3)	7·3 (3)	5·7 (6)
ClO_3^-	7·3 (4)	6·9 (1)	6·4 (2)	6·1 (1)	6·0 (1)
HCO_2^-	7·3 (1)	7·2 (2)	7·0 (8)	3·6 (1)	2·9 (2)

Figures are mean values of the algebraic differences of the IPSP sizes before and just after the injection, the number of trials being indicated in parentheses. For each anion the results on five motoneurones are shown in *a*, *b*, *c*, *d* and *e*. A different set of 5 motoneurones is of course employed for displaying the effects of each anion. Currents for the injection are 5×10^{-9} A during 60 sec in all but one case (*a* for NO_2^- ions, *). In this case 3×10^{-9} A was used in all of three injections. In another trial by 5×10^{-9} A in the same cell gave an IPSP as large as 20 mV.

that the effect of N_3^- ions is the same as that of other anion species of Table 3.

ClO_4^- ions are also effective, though the injection is technically difficult. During injection of this ion species through an electrode filled with $NaClO_4$ at a concentration from 5 to 1 M, the resistance of the electrode usually rose up to several thousand MΩ, with the consequence that it was impossible to record the IPSP and the resting potential. Moreover, when this block of the electrode happened early during the current application, the injection of anions was obstructed. Though this type of blockage sometimes occurred with electrodes filled with other anions, it was much commoner with the $NaClO_4$ electrodes, particularly when a hyperpolarizing current was passed in the intracellular position. Probably K^+ ions enter the electrode and precipitate in the tip as $KClO_4$, which has a low solubility (26 m-mole/100 g saturated solution at 40 °C; see Seidell, 1940). Indeed, a similar block was produced by a hyperpolarizing current when the electrode tip was in a 150 mM-KCl solution. Despite these difficulties it was possible to observe the reversal of the IPSP after injection into six cells listed in Table 2. The recovery of the IPSP followed an exponential curve reasonably well, though in only one case could the resting potential be properly controlled subsequent to the injection.

The effect upon the IPSP of the above anions, i.e., the increase of the IPSP in the depolarizing direction and its exponential recovery, leads immediately to the conclusion that they penetrate the activated inhibitory post-synaptic membrane, and therefore are labelled by plus signs in Table 5.

Non-penetrating anions

The effect of injection of BrO_3^-, HSO_3^-, $C_2O_4^{2-}$, $CH_3CH_2CO_2^-$, $CH_3(CH_2)_2CO_2^-$, $Fe(CN)_6^{3-}$ and $Fe(CN)_6^{4-}$ ions was similar to that described for SO_4^{2-} ions (Coombs *et al.* 1955*a*). The number of trials for each anion is shown in Table 2. After their injection the IPSP remained nearly the same or was slightly increased in size. The increased IPSP recovered gradually in about 1 min, as is illustrated for BrO_3^- ions in Fig. 6A. The increase of the hyperpolarization of the IPSP may be accounted for partly by the slight reduction of the resting potential which often followed the injection, and partly by the depletion of Cl^- ions from the cell by the hyperpolarizing current.

The ineffectiveness of the injection of the above anions immediately leads to the conclusion that the activated inhibitory post-synaptic membrane is impermeable to them (cf. Coombs *et al.* 1955*a*), except for HSO_3^- ions. Since the second hydrogen of H_2SO_3 has a relatively large acid dissociation constant, a conversion of HSO_3^- to SO_3^{2-} would occur in the cell, its degree being determined by the pH of the internal medium of the cell

that would be kept constant by a buffer action of the medium. According to the recent estimates of this dissociation constant, $10^{-6.99}$ to $10^{-7.205}$ (cf. Bjerrum, Schwarzenbach & Sillén, 1958), the ratio of $[SO_3^{2-}]:[HSO_3^-]$ in the cell would be expected to be close to unity at pH 7. Although there is no direct information on the pH of the internal medium of cat motoneurones, recent investigations on invertebrate nerve and muscle fibres (Caldwell, 1958) and on frog muscle fibres (Kostyuk & Sorokia, 1960) indicate that the pH in the cell is close to 7 under normal conditions. With the possible extreme of variation in pH, $\pm 0{\cdot}4$ (Caldwell, 1958; cf. also

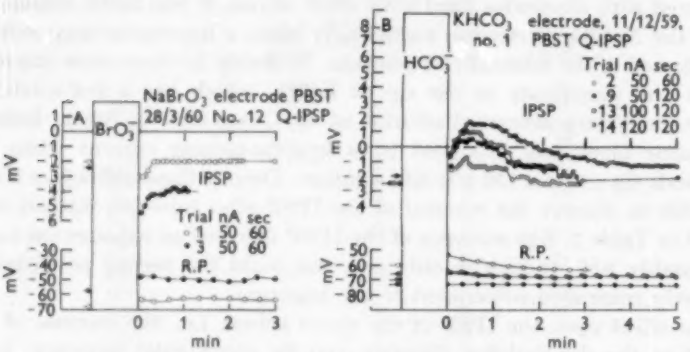

Fig. 6. Plot of IPSPs and resting potentials for injections of BrO_3^- (A) and HCO_3^- (B). Conventions as in Fig. 3.

Spyropoulous & Tasaki, 1960), the ratio of $[SO_3^{2-}]:[HSO_3^-]$ would decrease or increase by a factor of 2·5. Consequently, after relatively large injections by the passage of current of $5-10\times10^{-8}$A for 60–120 sec, the concentration of both HSO_3^- and SO_3^{2-} in the cell would be expected to be high enough to exhibit any influence that they might have upon the IPSP. The ineffectiveness of injection of HSO_3^- ions may thus be taken as indicating that neither HSO_3^- nor SO_3^{2-} ions pass through the activated inhibitory post-synaptic membrane. Similar considerations apply to the equilibrium between $H_2PO_4^-$ and HPO_4^{2-}, for which the acid dissociation constant is evaluated near 10^{-7} (cf. Bjerrum *et al.* 1958). The results obtained by Coombs *et al.* (1955*a*) by injecting HPO_4^{2-} can then be extended to $H_2PO_4^-$; hence the activated post-synaptic membrane is permeable to neither of these ions. For other anions (except HCO_3^-, see below) dealt with in this section, the values of the acid dissociation constants ensure their almost complete dissociation in the cell following injection.

F^- ions were also ineffective in converting the IPSP into a depolarizing potential, though a complication was introduced by their depolarizing action upon the resting membrane (Shanes & Brown, 1942). For example,

in four cells one injection by a current of 5×10^{-8} A for 60 sec sufficed to reduce the membrane potential down to -10 to -30 mV. However, in seven cases out of nine cells, where a relatively small current (3×10^{-8} A for 60 sec) was employed for injection of F^- ions, the resting potential was maintained at more than -50 mV. In these cases the IPSP was increased in the hyperpolarizing direction, indicating that the equilibrium potential of the IPSP was not significantly altered by injections. This situation closely resembled that with acetate ions, the ineffectiveness of which was proved on a more quantitative basis by the aid of IPSP–membrane–potential curve (Coombs *et al.* 1955*a*). In general, injection of penetrating anions by currents of 3×10^{-8} A induces a noticeable change of the IPSP. It can, therefore, be concluded that the activated inhibitory post-synaptic membrane is impermeable to F^- ions.

In their investigation Coombs *et al.* (1955*a*) could not exclude the possibility that HCO_3^- ions were slightly effective in changing the IPSP in the depolarizing direction. This ion species was therefore carefully tested on 23 occasions. The change induced in a PBST cell, in which eleven successful injections were made, is illustrated in Fig. 6B. In the first series of five injections a small reduction of the IPSP was observed, and as a typical and unique feature for this ion species the reduction was progressive with a maximum at about 30 sec after the injection, followed by a gradual recovery to the original size. In the later series the IPSP turned into a depolarizing potential following a similar time course to that in the initial series, but it never exceeded 4 mV. In all other cells only a small reduction of the IPSP was observed, without its reversal. These relatively small changes in IPSP cannot be explained by the conversion of HCO_3^- ions to H_2CO_3, for this would effectively remove only one fourth of the injected HCO_3^- at the pH of about 7 (see above and Bjerrum *et al.* 1958). Furthermore, the characteristic delayed maximum of action on the IPSP suggests that HCO_3^- ions are not effective by themselves and that the change of the IPSP is caused by some secondary process following the injection. One possibility to consider is a reduction of HCO_3^- to HCO_2^- ions in the cell, which is possibly facilitated by the high concentration of the former. The conversion of carbon dioxide to formic acid is catalysed by an enzyme which so far has only been reported to occur in certain bacteria (cf. Sumner & Myrbäck, 1952).

Since a light reduction of the hyperpolarizing IPSP following the injection of glutamate ions was described by Coombs *et al.* (1955*a*), this ion species also was re-examined with relatively large injections. However there was neither reversal nor any significant reduction of the IPSP. Thus, despite the very small effect previously reported, this ion species may be placed in the group of non-penetrating anions.

The sodium or potassium citrate electrodes have already been used for investigations on crustacean muscle (Boistel & Fatt, 1958) and on motoneurones (Kuno, 1959; Araki, Eccles & Ito, 1961) as indifferent for the IPSP. As would be expected, the injection of citrate ions gave results corresponding precisely to those given by the above non-penetrating anions.

In summary, sixteen anions, labelled by minus signs in Table 5, are specified as unable to penetrate the activated inhibitory post-synaptic membrane.

Untestable anions

With fourteen anion species, which have the hydrated size in the range of the present interest, injections were either unsuccessful or not tried because of the following reasons. They are labelled by *X* in Table 5.

(*a*) *Solubility of potassium salts.* The difficulty of injections due to low solubility of the potassium salts was described above (p. 419) for ClO_4^- ions. In this respect ReO_4^- and picrate ions were rejected as untestable, because their potassium salts have a solubility as small as 7·67 m-mole/100 g water at 40 °C and 2·89 m-mole/100 g water at 30 °C, respectively (cf. Seidell, 1952).

(*b*) *Oxidizing agents.* Blocking was regularly observed with electrodes containing MnO_4^- ions as either the sodium or potassium salt. A possible explanation is offered by their powerful oxidizing effect (cf. Latimer, 1959) which might cause coagulation of protein around the tip of the electrode. This kind of block happened even with electrodes having a large tip 2–3 μ across and made all attempts to inject these small ions (see Table 5) unsuccessful. Moreover, if injected successfully, it may be expected that the cell is damaged seriously by this ion species, as $KMnO_4$ solution of concentration as small as 50 mM (0·6 %) suffices to fix the tissue for electron microscopy (Luft, 1956). Similar difficulty due to the oxidation can be expected also with ClO_3^- ions, which, therefore, were not tested in the present experiments. With this ion species a further trouble would be introduced by a possible contamination of Cl^- ions. On the other hand, with BrO_3^- there was neither blocking of the electrode nor any sign of liberation of Br^- due to an oxidation process, as described above (p. 419), the effect of which would be readily detected as a change of the IPSP. The time-course of this oxidation reaction may be too slow to affect the IPSP in the time scale of the present experiments. Since the redox potentials for ClO_3^- and ClO_4^- ions are less negative than for BrO_3^-, the reduction of these ions, producing Cl^- ions, would not play any role in the observed IPSP change (pp. 412, 419).

(*c*) *Equilibrium with hydrogen ions.* Fourteen injections of CN^- ions were tried into six cells, but any effect upon the IPSP, which might be expected from their small hydrated size (Table 5), could not be observed. However, since HCN has a very small acid dissociation constant (near 10^{-9}, cf. Bjerrum *et al.* 1958), even large injections cannot be expected to cause any appreciable concentration of free CN^- ions in the cell (see p. 420). Therefore, it was not possible to determine if the flux of CN^- ions could contribute to the production of the IPSP. Similarly, CO_3^{2-} ions are untestable because of their small acid dissociation constant (near 10^{-10}, cf. Bjerrum *et al.* 1958). On the other hand, the dissociation constant for the second hydrogen of $H_2C_2O_4$ is large (more than 10^{-5}, Bjerrum, Schwarzenbach & Sillén, 1957); hence $HC_2O_4^-$ ions injected into the cell would be converted almost completely into $C_2O_4^{2-}$, so the former is untestable. Finally, seven anions, $(CN)_2N^-$, $(CN)_3C^-$, $S_2O_6^{2-}$, $(NO_2)_3C^-$, $CH_2FCO_2^-$, $CH_2CNCO_2^-$ and $CH_2ICO_2^-$, are excluded, because there are no available data about their acid dissociation constants (cf. Bjerrum *et al.* 1957, 1958).

Recovery of the IPSP after anion injection

With nine penetrating anions, Cl^-, Br^-, I^-, NO_2^-, NO_3^-, SCN^-, BF_4^-, ClO_3^- and HCO_2^-, the presumed exponential time course of the IPSP recovery was tested by logarithmic plotting of the IPSP sizes, which were measured as the algebraic difference from the values obtained at the end of the recovery. As is seen in Fig. 7C, the points obtained in three trials of NO_2^- injections into one and the same cell fall along the same straight line. In the cell of Fig. 7B the magnitude of the IPSP increased with each

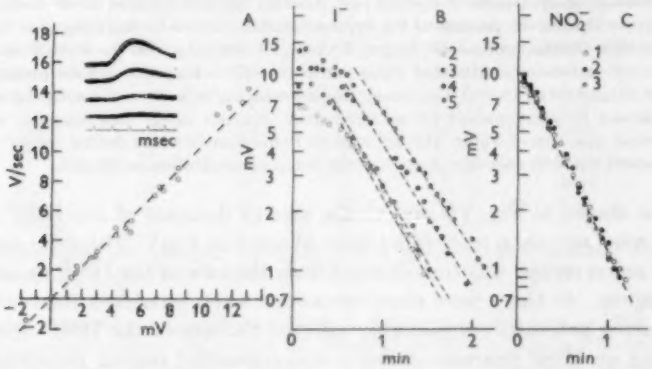

Fig. 7. A, illustrating a parallel change between the size and the steepest slope of rise of Ia IPSP by the injection of NO_2^- ions. Specimen records of recovering IPSPs are shown in the figure with a sweep velocity fast enough to measure the slope of rise. B, logarithmic plot of the IPSP size in the course of recovery after the injection of I^- ions. Note that ordinates are algebraic differences of IPSP sizes in the course of, and at the end of, the recovery. Plots are obtained from four trials in the cell that is illustrated in Fig. 3B. C, similar to B but for the injection of NO_2^- ions in a different cell from that shown in Fig. 3A.

injection of I^- ions, and for as long as 20–40 sec after the injection the slope of the recovery was much less steep than later. Nevertheless, after the late onset the recovery curves approximated to parallel straight lines. These curves of the IPSP recovery can be assumed to express the decrease of the intracellular concentration of injected ions as the ions diffuse out across the cell membrane into its environment (cf. Coombs *et al.* 1955*a*).

Over a wide range of values the size of the IPSP is proportional to the steepest slope of its rising phase (Fig. 7A) and this slope provides a measure of the maximum intensity of the inhibitory post-synaptic current (Curtis & Eccles, 1959). Thus the differences between the sizes of the IPSPs during the course and at the end of recovery, plotted in Fig. 7B and C, can be taken as indices of the intensities of the inhibitory post-synaptic current due to the efflux of injected ions throughout the various stages of recovery, which would in turn be proportional to the intracellular concentration of injected ions at that time. The size of the

Renshaw IPSP is measured at a fixed time (Fig. 1B) and exhibits changes resembling those of the Ia IPSP (Fig. 8C and D), when obtained in the same cell. Therefore it can be taken likewise as an index of the internal concentration of injected ions, despite the more complex nature of its generation.

Several explanations may be offered for the initial slow rate of recovery that is seen in Fig. 7B, for example. As a rule, following the injection of anions of Table 3, the resting potential was reduced by 5–15 mV and then recovered to a steady level within 30–60 sec (Figs. 3 and 4). This reduction of the resting potential will diminish the size of the depolarizing IPSP and this effect will decline as the resting potential recovers. Thus the decrease of the IPSP size in the initial period of the recovery would have been more rapid if the membrane potential had been kept constant. Furthermore, the lower resting potential would diminish the efflux of anions through the whole cell membrane and consequently the rate of the decrease in concentration of injected ions. Another factor to consider is the change of the cell volume. During the passage of the hyperpolarizing current for injecting ions it can be expected that cations, presumably largely K^+ ions, are carried across the surface membrane into the cell and consequently that water enters the cell to keep the osmotic pressure constant (cf. Coombs *et al.* 1955*a*). Conversely, anions escaping from the cell during the recovery phase should be accompanied by an equivalent quantity of K^+ ions together with an appropriate quantity of water. The cell volume would thus decrease during the ion escape, which would slow the rate of reduction of the anion concentration in the cell.

As is shown in Fig. 7B and C, the rate of decrease of the IPSP size is fairly constant when recovering from about 5 to 1 mV. The time constant of the anion escape was thus derived from the rate of the IPSP decrease in this region. As there were many occasions when a random fluctuation of the resting potential considerably affected the size of the IPSP, the cases showing an IPSP recovery under a well-controlled resting potential were limited to 2–6 cells for each anion species. As will be realized from the description above (pp. 417–419), it was not possible to obtain any reliable values in the rate of recovery of the IPSP following injections of N_3^- and ClO_4^- ions. The time constants thus measured show a considerable range for any particular ion species (Table 4); nevertheless, there appear to be significant differences between the various anions. These differences correspond approximately with those in the half time obtained by Coombs *et al.* (1955*a*) with four anions: Br^-, 10–20; SCN^-, 15–20; Cl^-, 10–35; NO_3^-, 23–35 sec.

In the constant-field theory (Goldman, 1943; Hodgkin & Katz, 1949; cf. also Coombs *et al.* 1955*a*) the time constant of the anion escape is related not only to the permeability coefficient of the membrane but also to the ratio of the cell volume to the surface area, and to the membrane potential. However, the cells employed in the calculations of Table 4 had large resting potentials (from -60 to -80 mV) and tolerated the penetration and injection for a considerable time, which suggests that they were all large cells. Thus the modification of the time constant arising from variations in the volume-to-surface ratio of the cell and in the membrane potential would be of little significance. Consequently, the time constant of the IPSP recovery can be assumed as reciprocally proportional to the

permeability coefficient through the whole surface area of the motoneurone. The relative values of the latter are shown in Table 4. When determining the time constant, the IPSP was observed at a repetition rate of 5/sec, and therefore it may be questioned how far the above-determined permeability coefficients involve those of the activated inhibitory post-synaptic membrane. However, the following calculations would indicate that the leakage of ions through the activated inhibitory patches is negligible.

TABLE 4. Time constant of IPSP recovery

Anion species	No. of cells	No. of trials	Mean time constant (sec)	Range (sec)	Relative ability to penetrate
Br^-	4	10	21	19–22	1·24
SCN^-	5	16	21	17–24	1·24
Cl^-	3	8	26	24–30	1·00
NO_3^-	6	15	28	22–41	0·93
BF_4^-	5	19	33	18–47	0·79
I^-	4	14	39	32–45	0·67
NO_2^-	3	11	42	40–44	0·62
ClO_3^-	2	4	63	60–66	0·41
HCO_2^-	2	6	90	88–92	0·29

The relative ability to penetrate is obtained from reciprocals of time constants. The value for Cl^- ions is taken as unity.

The peak value of the inhibitory post-synaptic current generating each Ia IPSP can be derived approximately from the steepest rising slope of the IPSP (cf. Curtis & Eccles, 1959), on the assumption that the total effective capacity of the motoneurone membrane is $2{\cdot}5 \times 10^{-9}$F (Coombs, Curtis & Eccles, 1959). The difference of the inhibitory current at a stage of the recovery following injection from that after the complete recovery can be assumed to be due to the injected ions at the former stage. The efflux of injected ions through the activated inhibitory patches is then obtainable by averaging the inhibitory current over the 2 msec of the activation for each Ia IPSP (Curtis & Eccles, 1959, Fig. 2), and further over the 200 msec of the repetition period. The integral of the average efflux over the whole course of the recovery yields the total quantity of ions ejected through the inhibitory patches. For example, this is calculated as 0·056 p-equiv in the case of Fig. 7A and 0·072 p-equiv in that of Fig. 8A. On the other hand, the passage of a current of 5×10^{-8}A for 60 sec could carry about 30 p-equiv of ions into the cell, and at least one fourth of them would remain at the end of the injection (Coombs *et al.* 1955*a*), and would then diffuse out of the cell during the course of the recovery. Consequently the activated patches of the inhibitory post-synaptic membrane would contribute to no more than 1% of the total efflux of injected ions from the cell.

Support for the above calculation is provided by the following experiment. In four successive injections of HCO_2^- ions into one and the same PBST cell having a large Renshaw IPSP together with the Ia IPSP, the IPSP recovery was determined with different combinations of synaptic potentials on sweeps repeated at five/sec: (1) Ia IPSP + EPSP; (2) Renshaw IPSP + EPSP; (3) Ia IPSP + Renshaw IPSP; (4) Ia IPSP + Renshaw ISPP + EPSP. In the series (1) and (2) the efflux of HCO_2^- ions through

the inhibitory patches should have been somewhat different because of the different durations of the active phase and of the different number of patches involved, and further, in the series (4) the ionic efflux through the inhibitory post-synaptic membrane should be the sum of those in (1) and (2). Among these series, however, there is no significant difference in the rate of the IPSP recovery, which is indicated by the slope of interrupted lines in Fig. 8 A, B and D, the time constant ranging from 80 to 88 sec. Similarly, comparison of the series (3) with (4) reveals that the EPSP repeated with the same rate as the IPSP does not influence the time course

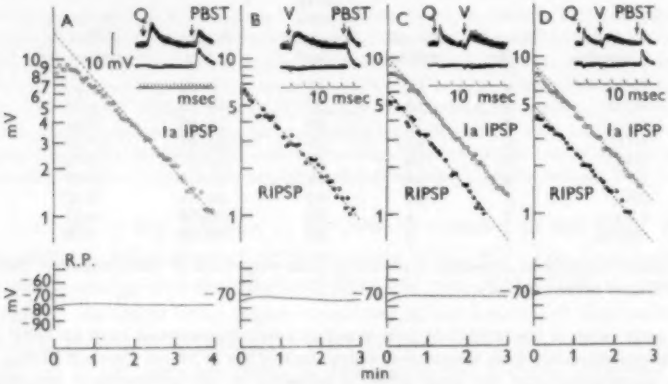

Fig. 8. Comparison of the time course of the IPSP recovery under operation of Ia IPSP, Renshaw IPSP and EPSP in different combinations. 7th (A), 8th (B), 9th (C) and 10th (D) trials of HCO_3^- injections into the same PBST motoneurone as shown in Fig. 5A. ○, size of Ia IPSP from quadriceps afferent (Q); ●, size of Renshaw IPSP (RIPSP) induced by a L7 ventral root stimulation (V), which was just subthreshold for the axon belonging to the cell. Note that ordinates are differential sizes of IPSPs in logarithmic scale as in Fig. 7B and C. Specimen records were obtained just (upper traces), and about 6 min (lower traces), after injections by the passage of current of 5×10^{-9}A for 60 sec. EPSPs were evoked from posterior biceps-semitendinosus afferents (PBST).

of the IPSP recovery. In other cases a spike potential was combined with the IPSP without any appreciable modification of the IPSP recovery. Besides the artificially induced synaptic potentials the motoneurones receive a random bombardment through its synapses. Indeed, Fig. 2 (A, B and E) shows a remarkable increase of the noise in the base line following the injection of ions, which apparently indicates the presence of random IPSPs. As is shown in Table 4, the time constant of the IPSP recovery varied over a considerable range for each anion species, but it was not possible to correlate it to the noise level in each cell. The contribution of the leaking current through randomly activated subsynaptic membrane

thus does not seem significant in modifying the IPSP recovery beyond the error of measurement in the present experiments. It can be concluded that the activated inhibitory post-synaptic membrane forms an insignificant part of the surface membrane of the motoneurones for which the relative permeability to anions is determined in Table 4.

Among the penetrating anion species HCO_2^- could possibly be destroyed by oxidation in the cell. The hydrogenlyase found in certain bacteria catalyses reversibly the conversion of formic acid into CO_2 and H_2 (cf. Sumner & Myrbäck, 1952). The relative ability of HCO_2^- ions to penetrate through the cell membrane would then be smaller than that indicated by the relative value of the time constant of IPSP recovery. However, as this ion species is at the extreme upper end of the present sequence of time constants, this possibility does not detract from the assumed sequence of abilities to penetrate in the reversed order of the time constants (Table 4). An enzyme which oxidizes SCN^- to CN^- ions was reported in erythrocytes of man and certain other species (cf. Goodman & Gilman, 1955), but there are no data available about nerve cells.

Influence of anions on the EPSP and spike potential

Monosynaptic EPSPs were recorded by sampling intermittently or continuously in the sweeps that were used for displaying the IPSPs (Fig. 2). Figures 3 (A, B), 4 (A, B) and 5 (A) show that the EPSP remains unchanged or is transiently decreased in size following injection of anions which produces a large change of the IPSP. This behaviour of the EPSP is readily explained by a transient change in the resting potential. According to Coombs, Eccles & Fatt (1955*b*), the size of the EPSP is reduced with a decrease of the resting potential when the latter is relatively low, while when the resting potential is high it remains almost constant against small alterations of the resting level. A large decrease of the EPSP by injection of toxic anions (Figs. 5B) can similarly be accounted for by a heavy reduction of the resting potential.

The depression of the spike potential, as illustrated in Figs. 3A and 5B, can also be attributed to a reduction in the resting potential. No anion species influenced the spike potential without a change in the resting potential.

DISCUSSION

Table 5 lists forty-one anion species dealt with in Results in the order of their hydrated size relative to K^+ as calculated from the limiting conductance. The latter values in water at 25° C (except for BF_4^-) are available in compilations by Landolt-Börnstein (1936), Harned & Owen (1943) and Robinson & Stokes (1959). These anions are now classified into three categories; penetrating +, non-penetrating −, untestable *X*. It is notice-

able that the separation into two groups of penetrating and non-penetrating ions is attained sharply by their effectiveness on the IPSP, and that there are no transitional ions forming an intermediate zone. The possibility that HCO_3^- ions fall in this zone is excluded above (p. 421), because their small effect could be attributed to a secondary process induced by their injections in the cell.

Since Boyle & Conway's (1941) discussion, it has generally been accepted that the permeability of the muscle membrane is selective to small hydrated ions. Large hydrated ions, either cations or anions, pass the membrane only with difficulty (see also Hutter & Noble, 1960). The pore-structure hypothesis of the membrane was thus proposed; the exterior and interior media of the cell communicate through pores, and small hydrated

TABLE 5. Effects on the IPSP and some physical properties of anions

Ion	IPSP effect	Relative hydrated size	Hydration energy (kcal/g ion)	Naked ion size (Å)	Ion	IPSP effect	Relative hydrated size
CN^-	*X*	0·93	.	.	$(CN)_3C^-$	*X*	1·58
Br^-	+	0·94*	63	1·95	$(NO_2)_3C^-$	*X*	1·60
I^-	+	0·96*	49	2·16	HCO_3^-	–	1·65*
Cl^-	+	0·96*	67	1·81	$CH_2FCO_2^-$	*X*	1·66*
NO_3^-	+	1·02*	(64)	(1·32)	$CH_2CNCO_2^-$	*X*	1·69*
NO_2^-	+	1·03*	(55)	(1·98)	$CH_3CO_2^-$	–	1·80*
N_3^-	+	1·07*	.	.	$CH_2ICO_2^-$	*X*	1·81
ClO_4^-	+	1·09*	(54)	(2·92)	$HC_2O_4^-$	*X*	1·83†
SCN^-	+	1·11*	(45)	.	SO_4^{2-}	–	1·84*
BF_4^-	+	1·12‡	.	(2·88)	$C_2O_4^{2-}$	–	1·94*
ClO_3^-	+	1·14*	(61)	(2·16)	$H_2PO_4^-$	–	2·04
MnO_4^-	*X*	1·17	.	.	SO_3^{2-}	–	2·04
					$CH_3CH_2CO_2^-$	–	2·05*
BrO_3^-	–	1·32*	(68)	(2·38)	CO_3^{2-}	*X*	2·12*
F^-	–	1·33*	94	1·36	$Fe(CN)_6^{3-}$	–	2·19
ReO_4^-	*X*	1·34*	.	.	$CH_2(CH_2)_3CO_2^-$	–	2·25*
HCO_2^-	+	1·35*	.	(2·03)	$C_6H_2(NO_2)_3O^-$	*X*	2·41*
$(CN)_2N^-$	*X*	1·35	.	.	HPO_4^{2-}	–	2·58
ClO_2^-	*X*	1·41	.	.	$Fe(CN)_6^{4-}$	–	2·66*
HSO_3^-	–	1·47	.	.	Citrate	–	3·08
$S_2O_3^{2-}$	*X*	1·58	.	.	L-Glutamate	–	——

Relative hydrated sizes were calculated by Stokes's law from limiting conductances in water at 25 °C (*Robinson & Stokes, 1959; † Harned & Owen, 1943; others, Landolt-Börnstein, 1936) except that for BF_4^- (‡) which was derived from the Stokes radius in liquid sulphur dioxide relative to that of ClO_4^- in the same solvent (Lichtin & Pappas, 1957). Hydration energy for halide ions is obtained from Kortüm & Bockris (1951). Other values of hydration energy in parentheses were calculated from lyotropic numbers (Voet, 1937) with quoted values for halide ions as standard. Naked ion sizes are shown for halide ions by their crystal radii (Pauling, 1960) and for oxy-anions by 0·25 nr (in parentheses), where n is the number of arms extending from the central atom and r their length, i.e. the interatomic distance between the central atom and the surrounding oxygen atoms plus van der Waals radius of the oxygen atom (Coutour & Laidler, 1957). Values of 0·25 nr for BF_4^- and HCO_2^- ions were calculated in a similar way from the data given by Pauling (1956), employing for HCO_2^- the average length of two C–O and one C–H ligands as r.

ions thereby pass the membrane, while those larger than the pore size are all non-penetrating. No interference of ions with the pore wall is implied in the theory. The applicability of this hypothesis to the inhibitory post-synaptic membrane (Coombs *et al.* 1955*a*) can now be criticized on the basis of an extensive anion series. In Table 5 there are twelve anions with hydrated size finely graded around unity of K^+ ions, from 0·93 of CN^- to 1·17 of MnO_4^-, while a further increase of the hydrated size is obtained with a jump from 1·17 for MnO_4^- to 1·32 for BrO_3^-, as the dotted line in Table 5 indicates. With the exception of CN^- and MnO_4^-, which could not be successfully tested, all ions above the line in Table 5 were able to penetrate the inhibitory subsynaptic membrane. On the other hand, ability to penetrate was displayed for only one species of anion, HCO_2^-, below this line, i.e., with hydrated sizes of 1·32 or higher. Evidently, HCO_2^- ions present the only inconsistency with the pore-structure hypothesis of the activated inhibitory post-synaptic membrane. This exception, however, might be accounted for by consideration of the following factors. One possibility is that the relative hydrated size of HCO_2^- ions is smaller at 38° C (the cat temperature) than at 25 °C. For example, the relative size of Na^+ ions against K^+ ions decreases from 1·47 at 25 °C to 1·43 at 35 °C, though the relative sizes of Cl^-, Br^-, I^- and NO_3^- are known to be much the same at 35 °C as at 25 °C (cf. Robinson & Stokes, 1959). There are, however, at present no data about the temperature effect on the limiting conductance of HCO_2^- ions. Another possibility is that the HCO_2^- ion has an ellipsoid rather than a spherical shape and hence may pass through pores smaller than the average diameter of these ions. It may be recalled that the relative permeability to Cl^-, Br^- and I^- ions through an artificial ion-exchange membrane is in the order of their polarizability, which makes these ions behave like rods (cf. Shanes, 1958). If the results can thus be reconciled with the pore-structure hypothesis, the size of pores in the activated inhibitory post-synaptic membrane can be determined as smaller than the hydrated size of BrO_3^- ions, yet larger than that of ClO_3^- ions. Though there is considerable discrepancy between the results of different methods of measuring the degree of hydration of ions or the absolute size of hydrated ions (see Bockris, 1949; Bell, 1958; Stern & Amis, 1959), values of 3·25 and 2·85Å will be adopted for the sizes of hydrated BrO_3^- and ClO_3^- ions respectively (Kielland, 1937). These values can be regarded as the upper and lower limits of the effective pore radius in the activated inhibitory post-synaptic membrane of cat spinal motoneurones.

The existence of pores such as assumed by Boyle & Conway (1941) has recently been demonstrated in the erythrocyte membrane by quite independent techniques (Solomon, 1960), the radius of pores being determined at 3·4–4·2Å. These pores are presumed to be charged positively,

which would account for the large difference between the permeabilities to Cl^- and K^+ ions with similar hydrated sizes (Table 5). On the activated inhibitory post-junctional membrane of the crustacean muscle Boistel & Fatt (1958) already adopted a similar assumption for explaining the differential permeability between Cl^- and K^+ ions. On the other hand, according to the recent comparative measurements of cation permeability in the muscle membrane (Conway & Moore, 1945; Lubin & Schneider, 1957; Sjodin, 1959; Mullins, 1959; Mullins & Moore, 1960) there is a large variation in the relative permeabilities to K^+, Rb^+, Cs^+ and Tl^+ in spite of their similar hydrated size. Since this is inexplicable by the pore-structure hypothesis, the theory has been modified by assuming that ions interact with the pore wall (Shanes, 1958; Mullins, 1959, 1960). The relative permeability would then be governed by a combination of factors such as hydration energy, naked ion size and statistical distribution of pore size. Unfortunately, as pointed out above (p. 426), it is not possible to evaluate accurately the relative effect of anions in the present experiments. Moreover, even when two different anions are injected into one and the same cell through a double-barrelled micro-electrode, the comparison of their effects is fraught with uncertainties, because the accumulation of anions during the injection would vary on account of the different rates of the ionic losses from the cell during the injection and also because of the possible differences in the transport numbers for the different ions being injected. Consequently it would be premature to discuss the possibility of interaction of ions with the pore wall in the activated inhibitory post-synaptic membrane. However, it should be pointed out that the exceptional behaviour of HCO_2^- ions in relation to the pore-structure hypothesis cannot be accounted for by assuming an interaction between ions and the pore wall after the manner suggested by Shanes (1958) or by Mullins (1959, 1960).

According to Mullins (1959, 1960) the permeability to ions is determined by the matching of their size in a dehydrated state with pore size, for which a Gaussian distribution is assumed tentatively; ions either too large or too small in their naked size fail to invade the membrane by replacing their hydration with the solvation obtained from the pore wall. However, it cannot be explained in this way that in the series of naked ion sizes (Table 5) the membrane is as permeable to small HCO_2^- ions as to large ClO_4^- or BF_4^-, but is impermeable to BrO_2^- which lies in between. By Shanes's (1958) hypothesis the impermeability to BrO_2^- or F^- ions should be attributed to their large hydration energy, because another limiting factor, the naked ion size, is smaller for them than for penetrating ClO_4^- and BF_4^-. For HCO_2^- ions the hydration energy is not given in Table 5, but the larger hydrated size, in comparison with BrO_2^-, in spite of the smaller naked size suggests a higher degree of hydration, and so a larger hydration energy. This is in conflict with the fact that HCO_2^- ions readily pass through the activated inhibitory post-synaptic membrane.

On the other hand, the relative anion permeability of the whole surface area of motoneurones was estimated from the relative values of the time

constant of the IPSP recovery (Table 4). Comparable figures are available from investigations on muscle membrane. Conway & Moore (1945) found that Cl^-, Br^- and NO_3^- ions penetrate the resting membrane in the ratio of 1:0·67:0·5. Abbott (quoted by Hill & MacPherson, 1954) reported a much slower escape of radioactive I^- from the muscle (time constant, 90 min) than of radioactive Cl^- (time constant, 10 min). Recently, Harris (1958) gave the following figures for time constants of escape of anions from muscle to normal Ringer's solution: Cl^-, 8–16 min; Br^-, 10–13 min; SCN^-, 46 min; I^-, 60 min; NO_3^-, 80 min; while Simon, Johnstone, Shankly & Shaw (1959) obtained a faster rate of escape of I^- ions than Harris (1958) found. Discrepancies between these authors are apparently due to the difference in the external ionic composition employed and in the method of loading muscles in solutions containing various anions. The present sequence of permeability (Table 4) is in reasonable agreement with the sequence presented by Harris (1958) except for the high permeability displayed for SCN^- ions. It is uncertain whether this discrepancy for SCN^- ions is due to the difference between the mammalian spinal motoneurones and frog muscles either in respect of the membrane itself or of the metabolic process destroying SCN^- ions within the cell (see p. 427).

With the series of nine anions of Table 4, it is interesting to discuss the validity of various hypothesis for explaining the order of their relative permeability in the whole surface of the motoneurone. First, from the point of view of Boyle & Conway's theory (1941), the slow penetration of ClO_3^- and HCO_2^- ions is in keeping with their large hydrated size. However, as pointed out by Shanes (1958), the much lower permeability for I^- relative to Cl^-, Br^- or even NO_2^- cannot be explained on the same basis, because their hydrated sizes are very close to each other (Table 5). Secondly, for the polyatomic ions NO_2^-, NO_3^-, and ClO_3^- the permeability is inversely related to the naked ion size, but this relation is not obeyed by the ions HCO_2^- and BF_4^- (Table 5). This is not explicable even by assuming a normal distribution of the pore size (Mullins, 1959, 1960). The third factor to consider is the hydration energy of ions. There has been a series of attempts to correlate the anion effects on the muscle, which would in turn relate to the anion permeability, with the well-known lyotropic series (Chao, 1934, 1935; Hill & MacPherson, 1954; Harris, 1958; Hutter & Padsha, 1959). The importance of the adsorbability of ions to the membrane, in terms of the lyotropy, has thus been emphasized, as influencing their penetration through the membrane (see Harris, 1956). The lyotropic series of anions follows the reversed order of their hydration energy (Voet, 1937), which is given in Table 5; $Cl^- > NO_2^- > Br^- > ClO_3^- > NO_3^- > I^- > SCN^-$. Even though the most serious inconsistency by SCN^- ions is excluded by the possibility of their chemical destruction (see p. 427), the

penetration of ClO_3^- which is slower than NO_3^- or even I^- does not agree with this sequence.

In short, it is not possible to account for the present sequence of relative permeability by any one of these factors, but perhaps an appropriate combination of them may be more successful. By Shanes's (1958) hypothesis deviations at the position of HCO_2^- as well as of BF_4^- in the relation between the relative permeability and the naked ion size (see above) could be adjusted by taking the hydration energy into account. As previously mentioned, the hydration energy of HCO_2^- should be large, at least more than that for BrO_3^-, so that the slow penetration in spite of the small naked size may be expected. In comparison with ClO_3^-, BF_4^- would have a smaller degree of hydration, and so a smaller hydration energy, because of the larger naked ion size against the presumed similar hydrated size (see legend of Table 5). This could then be the cause of the relatively quick penetration of BF_4^-. However, an exact weighted relation between the hydration energy and the naked ion size in determining the permeability would be necessary for establishing this hypothesis.

SUMMARY

1. Single micro-electrodes filled with solutions containing various anions were inserted into cat motoneurones in the lower lumbar segments and used both for electrophoretic injection of anions and for intracellular recording.

2. The effect of injections was appraised by changes in IPSPs, especially Ia IPSPs in PBST motoneurones, but also Renshaw IPSPs or polysynaptic IPSPs.

3. As revealed by the reversal and subsequent recovery of IPSPs, the activated inhibitory post-synaptic membrane was found to be permeable to seven anion species, I^-, NO_2^-, BF_4^-, ClO_3^-, ClO_4^-, HCO_2^- and N_3^-, in addition to those previously reported, Cl^-, Br^-, SCN^- and NO_3^-.

4. Eleven new anion species were included in the group of anions unable to penetrate the activated inhibitory post-synaptic membrane, while fourteen anions remained untestable.

5. The time constants of the IPSP recovery after injections were assessed for nine penetrating anions, by which a sequence was determined for the order of their relative ability to penetrate the whole cell membrane; $Br^- = SCN^- > Cl^- > NO_2^- > BF_4^- > I^- > NO_3^- > ClO_3^- > HCO_2^-$.

6. No anion species was found to affect EPSPs and spike potentials primarily, any effect appearing to be secondary to a depression of the membrane potential.

7. The ability of anions to penetrate the activated inhibitory post-synaptic membrane was related to their relative hydrated sizes and was

reconcilable with the pore-structure hypothesis of the activated inhibitory post-synaptic membrane.

8. The sequence of the relative permeability to anions through the whole cell membrane was compared with that obtained in the muscle membrane. The applicability of various hypotheses was discussed.

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THE ACTIVITY OF PULMONARY STRETCH RECEPTORS DURING BRONCHOCONSTRICTION, PULMONARY OEDEMA, ATELECTASIS AND BREATHING AGAINST A RESISTANCE

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Adrian (1933) concluded that pulmonary stretch receptors were primarily responsive to the volume of gas in the lungs. While this is generally accepted, subsequent investigations have shown that their activity is modified by other factors. Weidmann & Bucher (1951) found that they responded to changes in intrapleural pressure when the trachea was closed, Marshall & Widdicombe (1958) and Constantin (1959) showed that their activity was increased by pulmonary congestion, and Davis, Fowler & Lambert (1956) that the rate of change of lung volume influenced their discharge. Many pulmonary stretch receptors have a cardiac modulation of their discharge at low frequencies of firing, and many are stimulated by forcible deflation of the lungs (Widdicombe, 1954*b*).

Bronchial tone is a possible influence which has not been adequately studied in this respect. Intravenous injections of drugs causing bronchoconstriction lead to sensitization of pulmonary stretch receptors excited by positive pressure inflations (Widdicombe, 1954*c*) but these drugs also cause changes in the pulmonary circulation which might explain the changes in discharge frequency. Whitteridge (1948) found that inhalation of phosgene caused no change in the behaviour of pulmonary stretch receptors, but the mechanical properties of the lungs were not assessed, so it is uncertain if bronchoconstriction occurred. This paper describes a re-investigation of this problem; it is important partly because the receptors are thought to lie in the air-passages and therefore a response to alterations in bronchial muscle tone might be expected, and partly because a possible role for the pulmonary stretch receptors is to signal changes in the physical state of the lungs and thus to adjust the pattern of breathing to be mechanically economical (Otis, Fenn & Rahn, 1950; Christie, 1953) or to minimize respiratory muscle force (Mead, 1960).

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As well as bronchoconstriction, the addition of resistances external to the airway, pulmonary oedema and atelectasis have been tested on pulmonary stretch receptor activity.

METHODS

The anaesthetic was pentobarbitone sodium (Nembutal, Abbott Laboratories Ltd, 32 mg/kg) given intraperitoneally to cats, and intravenously to rabbits. Intra-oesophageal pressure was recorded with a condenser manometer connected to a saline-filled catheter; changes in this pressure were considered equivalent to changes in intrapleural pressure. The frequency response of the recording system was better than 200 c/s. Air flow was recorded from a gauze-screen pneumotachograph similar to, but smaller than, that described by Lilley (1950) for man. Lung volume changes were obtained by planimetric integration of the air-flow record, or by enclosing the animal in a trunk plethysmograph (Dawes, Mott & Widdicombe, 1951), and measuring with a condenser manometer the small pressure changes in the closed system.

Action potentials were recorded from single active nerve fibres in the peripheral end of a cut cervical vagus nerve. Small strands were dissected from the nerve, placed on saline-wick or platinum electrodes, and their electrical activity amplified, displayed on a cathode-ray oscilloscope and recorded in conventional manner. All the pulmonary stretch receptors studied were slowly adapting with low thresholds (Adrian, 1933).

Bronchoconstriction was induced by administration of an aerosol of a 2–5% solution of histamine acid phosphate from a clinical 'atomizer'; the animals inhaled the aerosol during spontaneous breathing for sufficient time (15–50 breaths) to cause bronchoconstriction. Lung resistance (total viscous resistance to flow) was calculated from analysis of the transpulmonary pressure, air flow, and lung volume records (Crosfill & Widdicombe, 1961). Lung compliance was also determined from these records, and from the transpulmonary pressure changes during lung inflations to known volumes with a syringe.

Pulmonary oedema was produced in rabbits by intravenous injection of adrenaline sulphate (1–4 mg) and was detected by the appearance of froth in the trachea. With cats oedema was caused by rapid intravenous infusion of large volumes of isotonic saline (up to 200 ml.).

Pulmonary collapse was induced in the left lung of cats whose life was maintained by breathing with the right lung. Through a lateral chest opening the right main bronchus was cannulated and the attached tube passed through the chest wall, which was then closed to allow spontaneous breathing (Widdicombe, 1954*a*). The stump of the right main bronchus was ligated. The left lung was allowed to breath 100% O_2 through the tracheal cannula, and after 5–10 min the cannula was closed. Over the next five minutes the O_2 was absorbed and the left lung collapsed completely; this was confirmed subsequently by repeating the procedure and opening the chest before re-inflation.

The lungs were inflated either to constant volumes with a syringe, or to constant pressures from a pressurized tank or from a weighted bag. Inflations with a syringe were usually progressive in steps of 20 ml. up to 100 ml., but the inflations were sometimes interrupted with the lung volume returning to resting level between inflations. In some experiments cats were enclosed from the neck down in a trunk-plethysmograph and the lungs inflated by connecting the box to a negative-pressure tank or by withdrawing air from the box with a syringe.

The Hering-Breuer reflex was assessed in animals with intact vagi by inflating the lungs and determining the ratio of the apnoeic pause during inflation divided by the average length of the respiratory cycle before inflation.

RESULTS

Pulmonary collapse. This was studied with eight pulmonary stretch receptors from four cats and a rabbit (Table 1). During the collapse five of the receptors began to discharge or increased their spontaneous discharge; three of these had spontaneous activity at the resting lung volume, and in these the discharge rate increased. On re-inflation of the collapsed lung all but two of the end-organs were stimulated by far smaller volumes than previously, in spite of the fact that the lung gas volume had been decreased

TABLE 1. Activity from pulmonary stretch receptors during lung oedema and collapse

	Response				
Receptor	At resting lung volume	On forced deflation	During collapse	During oedema	On re-inflation
58A	0	0	0	.	Inhibited
58B	0	0	0	.	Sensitized
59A	0	0	+	.	Inhibited
59B	0	0	+, irreg.	.	Sensitized
*62D	0	+	0	.	Sensitized
63A	Active	+	+	.	Sensitized
63C	Active	0	+	.	Sensitized
63D	Active	++	+, irreg.	.	Sensitized
*114A	0	0	.	0	Inhibited
113D	0	+	.	+	Sensitized
53D	Active	0	.	+	Sensitized
115B	Active	+	.	0	Sensitized

+, ++; small and large increases in discharge frequency compared with the discharge rate at resting lung volume. Irreg.; irregular discharge pattern. Inhibited and sensitized refer to the response to inflations with a syringe to constant volumes. * = rabbit.

nearly to zero. In some instances very small volumes of air (1–5 ml.) caused rapid discharges, whereas the same inflation would have caused only a small increase in frequency in aerated lung (Fig. 1). The discharge adapted more rapidly than in the previous controls, and the volume–frequency curves were asymptotic (Fig. 2); this prevents a quantitative assessment of the sensitization. Two endings were not stimulated by inflation of the lungs until the lobes were nearly completely aerated, when the receptors suddenly started to discharge rapidly. On complete aeration of the lobes all the receptors took up their control volume–discharge-frequency relationships (Fig. 2).

Pulmonary oedema. Only two cats and two rabbits were used for these experiments; the production of oedema kills the animal and only one receptor can be investigated in each individual. The behaviour of the receptors was similar to that after collapse. Three endings responded vigorously to very small inflations; for example receptor 115B was excited by inflations of 0·5 ml., whereas previously the threshold had been over

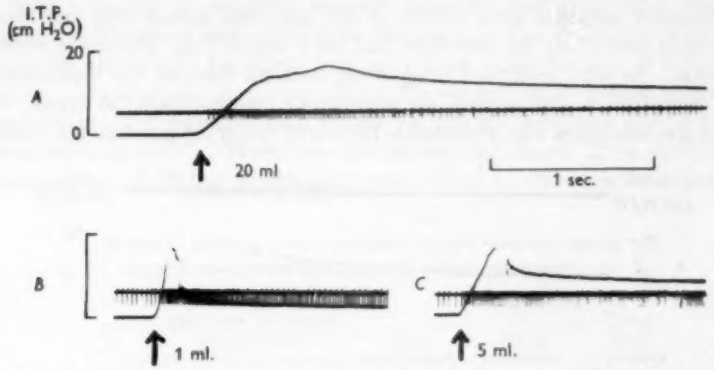

Fig. 1. Response of a pulmonary stretch receptor (59 B) before and during collapse of the lung. Upper trace: action potentials in left vagal nerve fibre. Lower trace: intratracheal pressure. *A*: 20 ml. inflation of the left lung at signal. The left lung was then collapsed. *B*: 1 ml. inflation of left lung. *C*: 5 ml. inflation of left lung. Collapse of the lung caused a spontaneous discharge from the receptor, and the small inflations produced a greater discharge frequency than the larger inflation before collapse. The nerve strand was readjusted on the electrodes between *A* and *B*.

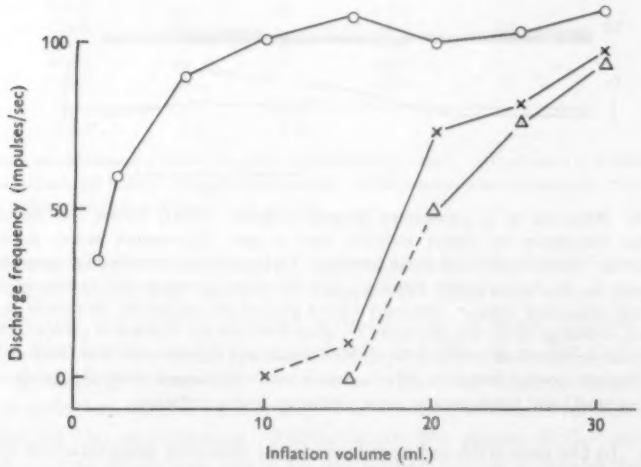

Fig. 2. Lung-volume–impulse-frequency curves for a pulmonary stretch receptor (62 D) before (△) and during (○) collapse of the left lung, and after re-inflation (×). Collapse caused a great sensitization at low lung volumes, and an asymptotic shape to the curve.

15 ml. from the expiratory level. One ending (114A) was not excited on subsequent inflation until 50 ml. of air had been forced into the lungs, although previously its threshold had been about 7 ml. above the resting volume. As with collapsed lung, when oedema lowered the threshold of the receptors it also caused an asymptotic volume/response curve, but since the condition was irreversible the curve never returned to the control

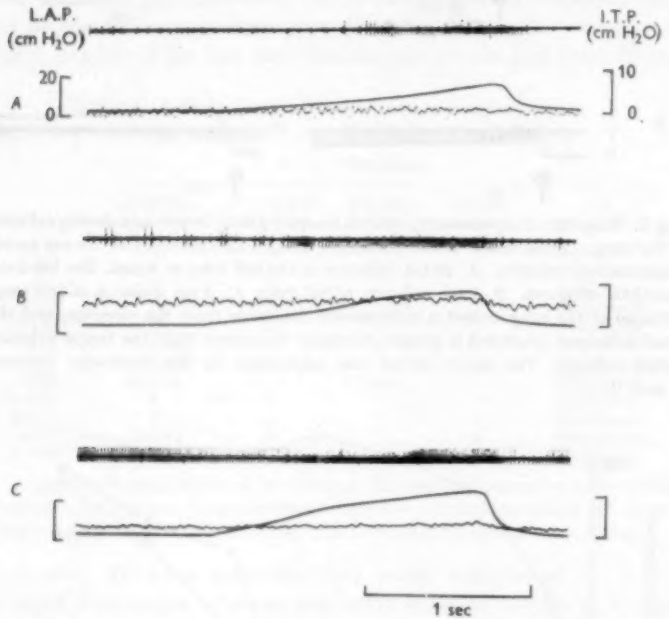

Fig. 3. Response of a pulmonary stretch receptor (113D) before and during oedema formation by saline infusion into a cat. Uppermost trace: action potentials. Middle trace: left atrial pressure. Lowest trace: intratracheal pressure. *A*: control. *B*: during saline infusion. The left atrial pressure and intratracheal pressure (constant volume inflations with a pump) are increased, and there is a greater discharge from the receptor. *C*: after the infusion oedema is present, the left atrial pressure has returned nearly to normal, and the intratracheal pressure is even higher during inflation. The receptor now discharges with the lungs at 'resting level' and has a greater peak discharge during inflation.

position. In the cats with saline infusions the receptor sensitization typical of pulmonary vascular congestion (Marshall & Widdicombe, 1958) was seen before the appearance of oedema fluid (Fig. 3), but the latter formed too rapidly in the rabbit to allow this intermediate phase to be seen.

Bronchoconstriction. Bronchoconstriction induced by inhalation of histamine aerosol was tested on fifteen pulmonary stretch receptors

(eleven from cats, four from rabbits). Eleven of these showed enhancement of their response to constant volume inflations and four little change (Table 2). Figures 4 and 5 illustrate the results. For inflations of 60 ml. the mean increase in discharge frequency 1 sec after the end of inflation was 21·1 impulses/sec, or 29·5% above the control value before bronchoconstriction. The corresponding values for 100 ml. inflations are 16·5 impulses/sec and 15·9%. These are statistically significant ($P < 0.01$) and sensitizations of similar significance were found at all levels of inflation.

TABLE 2. Activity from pulmonary stretch receptors during bronchoconstriction when compliance decreased

Receptor	Change in discharge frequency (impulses/sec) at 60 ml.	at 100 ml.	Lung compliance (% change)	Lung resistance (% change)	Lung time constant (% change)
33A	19	20	−12	47	39
35A	43	44	−17	258	198
46F	21	34	−23	217	145
47A	35	14	−11	150	122
47B	5	− 3	−12	127	100
47C	34	2	−14	163	126
57A	24	29	−43	473	327
57C	− 9	5	−36	195	198
57D	20	12	−42	325	146
57E	29	11	−15	70	45
*25A	17	16	−18	.	.
*45A	38	.	−25	72	29
*24C	2	6	−16	.	.
*24D	30	24	−15	.	.
*53D	9	.	−15	25	7
Mean	21·1	16·5	.	.	.
S.E.M.	3·8	3·4	.	.	.

Values are increases (positive) unless otherwise expressed. All values are differences from controls obtained before bronchoconstriction. Compliance and resistance were measured during spontaneous breathing. * = rabbit.

Sometimes the volume–response curves became asymptotic, suggesting that some pulmonary units had closed off. Thus the mean percentage sensitization was greater at small volumes than at large volumes.

This increase in discharge is probably not due to a rise in functional residual capacity because the increase in expiratory lung volume measured in four cats was only 3·4 ml. (range 1·2–3·9 ml.) which is far too small to account for the sensitization. Furthermore, the shape of the volume–response curves could not easily be accounted for by a rise in functional residual capacity which should merely elevate the curve (Fig. 5). The increases in airway resistance were from 25 to 473% of the control values (measured in seven of the nine animals); compliance always decreased (by down to −43% of the controls). Blood pressure and heart rate did not

appreciably change, although respiratory fluctuations in blood pressure became larger.

Inflations were also made at constant pressure, either positive or negative; inflation volumes decreased owing to the stiffening of the lung. For nine receptors in four cats, four showed a greater discharge frequency for given pressure inflations, two little change, and three a decrease in frequency. The mean change was not statistically significant from the controls.

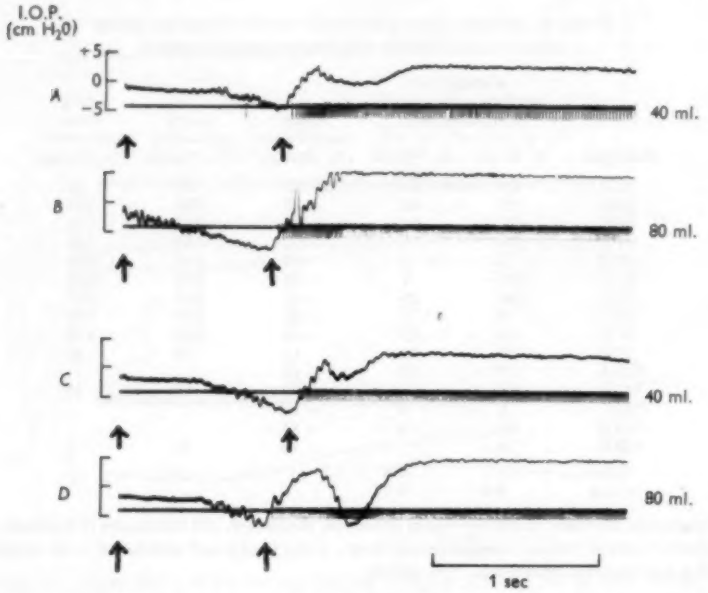

Fig. 4. The effect of bronchoconstriction caused by inhalation of histamine aerosol on the discharge of a pulmonary stretch receptor (47C). Upper trace: intra-oesophageal pressure. Lower trace: action potentials. At the first arrows the tracheal cannula was closed, at the second arrows the lungs were inflated with a syringe. *A* and *C* are 40 ml. inflations; *B* and *D* are 80 ml. inflations. Between *B* and *C* the cat inhaled 3% histamine aerosol for twenty-five breaths, which led to a greater discharge frequency at both lung volumes.

Attempts were made to obtain an increase in resistance to flow without change in compliance, as is possible with human beings inhaling histamine aerosol (DuBois & Dautrebande, 1958). This proved difficult with the cats, and it was successful for only six out of ten receptors (Table 3). With these the pulmonary resistance increased a mean of 69% (range 15–180%) and the impulse frequency increased a mean of 4 impulses/sec (range −15 to

+23 for 32 measurements at different inflation volumes). This increase is not statistically significant. Figure 5 illustrates a typical negative result.

The action of histamine aerosol was also tested on the Hering-Breuer inflation reflex (four cats, three rabbits). With constant-volume inflations the reflex was always enhanced, whether expressed as a ratio or as the absolute length of the apnoea (Fig. 6). With inflations to constant pressure,

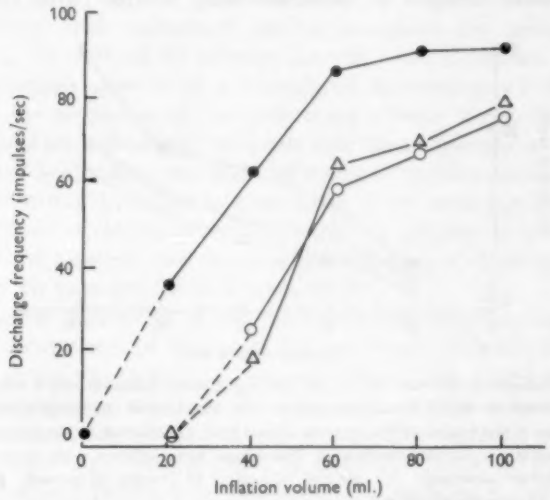

Fig. 5. Inflation-volume–discharge-frequency curves for a pulmonary stretch receptor (46F). △: control curve. ○: after 10 breaths of 5% histamine aerosol, which caused a 5% decrease in compliance, and a 72% increase in resistance. There is no significant change in the receptor activity. ●: after a further 20 breaths of histamine aerosol. The compliance was now decreased 23% and the resistance increased 217%. The receptor discharge is increased at each volume.

TABLE 3. Activity from pulmonary stretch receptors during bronchoconstriction when compliance did not greatly change

Receptor	Change in discharge frequency (impulses/sec) at 60 ml.	at 100 ml.	Lung compliance (% change)	Lung resistance (% change)	Lung time constant (% change)
46F	6	8	−5	72	64
47A	10	− 7	0	15	15
47B	−15	− 4	−5	33	27
47C	5	4	−8	60	47
57A	7	23	+3	180	188
57D	8	10	−4	54	48
Mean	3·5	5·6	.	.	.
S.E.M.	3·8	4·4	.	.	.

Values are expressed as in Table 2.

and therefore reduced volume, the reflex was reduced in two cats, potentiated in one and showed little change in four; expressed as the absolute length of the apnoea, two animals had shortened inhibitions, and the other five showed little change. There was no correlation with species.

Resistance breathing. Weidmann & Bucher (1951) have shown that many pulmonary stretch receptors modify their discharge rate when the intrapleural pressure changes at 'constant' lung volume (with the trachea

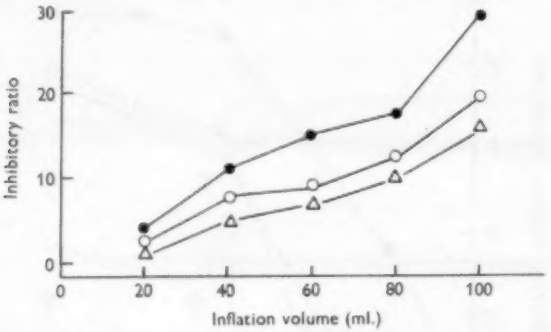

Fig. 6. Changes in the activity of the Hering–Breuer inflation reflex before and during inhalation of 3% histamine aerosol. The strength of the reflex is expressed as the ratio of the length of the apnoea caused by lung inflation to the length of the control respiratory cycles (ordinate). The lungs were inflated with a syringe to given volumes (abscissa). △: control. ○: after 15 breaths of aerosol. ●: after a further 15 breaths of aerosol.

occluded). This has been confirmed. Of twenty-two receptors, sixteen increased their activity when the intrapleural pressure became more negative under these conditions, four showed little change, and two were inhibited. For the sensitized receptors, when the lung volume was just below threshold each inspiratory effort caused a discharge; when the volume was well above threshold for the ending there was an increase in the frequency of the continuous discharge. 'Eupnoeic' inspiratory efforts caused an increase in the rate of firing and the absolute change in frequency varied little with the lung volume. For all twenty-two receptors there was a mean increase in discharge of 10·5 impulses/sec (S.E.M. 2·4) during inspiratory efforts against a closed trachea, which is statistically significant ($P < 0{\cdot}01$).

It was therefore expected that adding a resistance to the tracheal cannula would sensitize the majority of receptors for given volumes during spontaneous breathing. With a resistance of 13·2 cm H_2O/l./sec (about the cat's total lung resistance) three out of seven receptors were slightly sensitized, one inhibited, and three gave no clear change in response.

Since, however, adding a resistance slowed the rate of inspiration and therefore allowed longer for the receptor to adapt, it is possible that the three last-mentioned endings would have shown sensitization had the rate of inflation been as rapid as in the control breaths. Because of this difficulty in interpretation more receptors were not studied with this technique.

DISCUSSION

It is likely that pulmonary stretch receptors are 'smooth muscle spindles' in the walls of the airways (Larsell, 1922; Elftmann, 1943) since these end-organs alone have a histological appearance which correlates well with the properties of the pulmonary stretch fibres (Widdicombe, 1954*c*). These smooth muscle receptors lack the complexity of structure of skeletal muscle spindles, and their microscopic appearance does not indicate how they might respond to contraction of the smooth muscle in which they lie. Many of the receptors are excited by collapse as well as by distension of the airways, and this is especially true of those in the extrapulmonary air passages (Widdicombe, 1954*b*).

The changes in activity of pulmonary stretch receptors during atelectasis and oedema can be explained if the endings lie in the air passages. When the alveoli are collapsed or contain oedema fluid, a lung inflation will cause a disproportionate distension of the airways, since surface tension will hinder the entry of gas into the alveoli. The physical properties of collapsed lungs have been reviewed by Radford (1957) and by Clements, Brown & Johnson (1958); a critical pressure is required to open collapsed alveoli, and until this pressure is reached the insufflated air will remain in the air passages. The elastic pull of collapsed or oedematous alveoli will also affect airway diameter, and thus it is not surprising that the 'spontaneous' discharge of the receptors changed during the onset of both conditions; nor is it surprising that a few receptors were not stimulated by small inflations, for these endings may have been in collapsed or oedematous areas more resistant to inflation than the rest of the lung. Although expansion of the lung with oedema fluid could account for the increase in spontaneous discharge by some receptors, this distension is unlikely to explain the very great increase in impulse frequency caused by small (0·5–5 ml.) inflations of the lung. Thus the results with oedema and atelectasis can be explained if the receptors lie in the airways. The results also support this view, for the rather consistent sensitizations would be inexplicable if the end-organs were in the alveoli or pleura.

The action of inhaled histamine aerosol can be explained in a similar way. In earlier work (Widdicombe, 1954*c*) intravenous injections of histamine sensitized pulmonary stretch receptors, but it was subsequently established that pulmonary vascular congestion can increase the discharge

of the endings (Marshall & Widdicombe, 1958; Constantin, 1959). The intravenous histamine may have acted on the pulmonary vascular bed, thus decreasing lung compliance and causing greater distension of the airways. When histamine was given as an aerosol, systemic blood pressure and heart rate did not change, so it is unlikely that much histamine was absorbed into the circulation. Pulmonary stretch receptors were sensitized only when lung compliance decreased. The mechanism of this decreased compliance is not known. Bronchoconstriction exerts little tension longitudinal to the airways (Radford & Lefcoe, 1955) and does not change compliance in man provided pulmonary units are not closed (DuBois & Dautrebande, 1958). In the cats, therefore, there may have been collapse or oedema as well as constriction or the histamine may have acted on the pulmonary vascular bed. A decrease in compliance by whatever means would distend the airways and stimulate pulmonary stretch receptors both by direct pull on the bronchi and by redistribution of inspired air, exactly as has been suggested for pulmonary congestion, collapse and oedema. The lack of correlation between individual receptor responses and the changes in either lung resistance or compliance may be due to differences in the sites of receptors in the airways and to non-uniform distribution of bronchoconstriction.

The above explanation of the way histamine aerosol increased the lung volume-response of pulmonary stretch receptors is only valid if the receptors lie in the larger airways and if the main change in resistance to inflation after histamine takes place distal to this site, i.e. in the bronchioles and alveolar ducts. Under these circumstances a smooth muscle contraction in a large bronchus might be outweighed by the passive extra expansion of the airway due to the mechanical changes further down the airstream. When the bronchioles became constricted (and thus increased airway resistance) without closure of pulmonary units, then the explanation requires that the larger bronchi should have unchanged expansion (except during phasic changes of volume) so that bronchial stretch receptors in them would have unchanged activity. This explanation makes these assumptions: (*a*) that the receptors are in the larger bronchi, which is supported by histological investigations (e.g. Elftmann, 1943) if the endings are 'smooth muscle spindles'; (*b*) that in the cat inhalation of histamine aerosol constricts bronchioles, and that any contraction of the smooth muscle in the bronchi has little effect on the diameter of the latter airways; this is compatible with the facts that the bronchi are strongly supported by cartilage and that it proved difficult to cause an increase in resistance without concomitant decrease in compliance; (*c*) that the pulmonary stretch receptors respond to circumferential stretch of the bronchi but are less influenced by changes in contraction of the smooth

muscle in which they lie. It would follow that if smooth muscle contraction narrows an isolated bronchus then stretch receptors in its wall should be inhibited. This behaviour is probably true for pulmonary stretch endings (Widdicombe, 1954*c*), but an unequivocal experiment has not yet been done.

The above considerations may seem speculative, but they are supported by some observations of Folkow & Pappenheimer (1955), who found that intravenous or aerosol administration of histamine or pilocarpine decreased the compliance of cats' lungs with no change in anatomical dead space at given lung volumes. They concluded that the bronchoconstrictor drugs were acting on the small bronchioles with no constriction of the larger airways. Furthermore, Severinghaus & Stupfel (1955) found that in dogs Arfonad (a ganglion-blocking and histamine-releasing agent) caused a constriction of the terminal bronchioles with considerable (60 %) increase in anatomical dead space and, for one dog, that intravenous histamine caused a marked fall in compliance, an increase in physiological dead space (63–89 %) and a slight fall in anatomical dead space (9 %). These two groups of results support the suggestion given above as to the cause of the change in pulmonary stretch receptor discharge during bronchoconstriction.

In all these experiments haemodynamic changes in the pulmonary vascular bed have not been measured. Severe pulmonary congestion (left atrial pressures to 20–40 mm Hg) increases the discharge of pulmonary stretch receptors by about 20 % at given volumes. It is therefore possible that much of the action of histamine aerosol on pulmonary stretch receptors could be due to changes in the pulmonary vascular bed, although the decreases in compliance are far greater than would be expected from lung congestion alone (Hughes, May & Widdicombe, 1958).

The discharge frequency of most pulmonary stretch receptors increased when the intrapleural pressure fell with the trachea closed, an effect described by Weidmann & Bucher (1951). The changes in discharge were consistent, and therefore were probably not due to alterations in the shape of the lungs, for elongation of occluded lungs by pull of the diaphragm should stimulate and inhibit receptors in approximately equal proportions. The magnitude of the responses is compatible with increases in pulmonary vascular volume, but this has not been tested. With a resistance added to the tracheal cannula similar effects were seen, but they were difficult to assess owing to differences in the rate of pulmonary expansion and the adaptation rates of the receptors.

One object of this study was to test Christie's (1953) suggestion that pulmonary stretch receptors might adjust the cycle-to-cycle pattern of breathing in response to changes in the mechanical properties of the lungs. This possibility has been further discussed by Davis *et al.* (1956) and by Mead (1960). Otis *et al.* (1950) showed that for any given compliance,

resistance to flow, dead space and alveolar ventilation rate there was a frequency of breathing and corresponding tidal volume at which minimal work was performed on the respiratory system. Frequencies of breathing higher or lower than this optimal rate required greater energy for the same alveolar ventilation and thus would be uneconomical. Mead (1960) has recently suggested that it is not the work of moving the lungs which may be kept at a minimal value, but rather the mean respiratory muscle force. It is not necessary to discuss the relative merits of these two viewpoints, for they agree in the pertinent conclusion that the optimal rate of breathing depends on two factors, the ratio of alveolar ventilation rate to dead space volume, and the product of compliance and resistance to flow (i.e. time constant) of the respiratory apparatus (Mead, 1960). If the mechanical properties of the lungs change so as to increase the time constant then breathing frequency should slow, if the time constant decreases then the breathing frequency should speed. The problem is whether or not the pulmonary stretch receptors can mediate such changes in frequency.

Alterations in the mechanical behaviour of the lungs can affect the activity in pulmonary stretch receptors in two ways. The first is by changing the sensitivity of the endings to different lung volumes. Three conditions which stiffen the lungs and thus decrease their time constant—congestion, collapse and oedema—all increase discharge from pulmonary stretch receptors; this would tend to cut short inspiration, reduce tidal volume and increase the frequency of breathing (Adrian, 1933). Thus either the work of breathing or the mean respiratory muscle force might be prevented from rising in these conditions. By contrast, inhalation of histamine aerosol increased the time constant of the lungs (Tables 2 and 3; resistance rose more than compliance fell) and an inhibition of pulmonary stretch receptor discharge might be considered desirable to slow breathing to adjust it to the appropriate optimal value. In fact receptor discharge increased after histamine with constant-volume inflations.

In assessing the action of histamine, the second way in which changes in pulmonary mechanics can influence stretch receptor discharge must be considered. An alteration in the rate of expansion of the lungs will affect the rate of build-up of impulses in pulmonary stretch fibres going to the medulla. When the time constant of the lungs increases (as with histamine aerosol) the lungs will take longer to inflate in spontaneous breathing and the inhibitory influence of the pulmonary stretch receptors may be delayed. Furthermore, the peak discharge rate of the receptors is considerably decreased by slowing the rate of inflation to constant volume (Knowlton & Larrabee, 1946; Davis *et al.* 1956), so that the total number of impulses from pulmonary stretch receptors would be decreased as well as delayed. Whether in spontaneous breathing this inhibitory action would outweigh

the sensitization of pulmonary stretch receptors (to constant-volume inflations) after histamine is not known, but the time constant of the lungs can increase very greatly in bronchoconstriction (Table 2). Although the response of the receptors to bronchoconstriction during spontaneous breathing could be studied, the reflex respiratory behaviour would be far more difficult to assess. It would depend on the response of the respiratory areas of the brain to the timing as well as quantity of impulses from pulmonary stretch endings; this had received little study and would in any event almost certainly be affected by anaesthesia. It should also be emphasized that the Hering–Breuer reflex is only one component of a complicated assembly of pulmonary, cardiovascular and respiratory reflexes, all of which may adjust breathing in response to mechanical changes in the lungs. Furthermore, the resistance and compliance of the lungs are only part of the mechanical properties of the entire respiratory apparatus.

SUMMARY

1. The activity of pulmonary stretch receptors of cats and rabbits has been measured before, during and when possible after the production of pulmonary oedema, collapse, bronchoconstriction and breathing against a resistance. Lung-volume–discharge-frequency curves were plotted to determine the changes in sensitivity of the receptors.

2. During atelectasis of the left lung six out of eight receptors in that lung had a very great increase in sensitivity, being stimulated by inflation volumes (1–5 ml.) which had little or no effect before.

3. Pulmonary oedema similarly (but irreversibly) affected three of four receptors.

4. Bronchoconstriction caused by inhalation of histamine aerosol was tested on fifteen receptors. Eleven increased their response to inflations, and the mean change was statistically significant. This sensitization was only seen when enough aerosol was inhaled to decrease compliance as well as to increase resistance to flow.

5. Occlusion of the trachea increased the discharge of sixteen of twenty-two receptors during inspiratory efforts at all lung volumes.

6. These responses can be explained if the receptors are in the air passages and are sensitive to circumferential stretch. The results make other possible sites unlikely.

7. The results are discussed in relation to the hypothesis that pulmonary stretch receptors adjust the pattern of respiration from breath to breath in response to changing mechanical conditions in the lungs. It is concluded that such a role exists.

Some of the apparatus used in these experiments was bought by the Central Research Fund, University of London.

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INHIBITORY INFLUENCES ON PRIMARY AND SECONDARY CORTICAL PHOTIC POTENTIALS ORIGINATING IN THE LOWER BRAIN STEM

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In a recent communication (Palestini, Lifschitz & Armengol, 1959), a study of the habituation of primary and secondary cortical photic potentials in cats which had been subjected to a mid-pontine pre-trigeminal transection was reported. E.e.g. recordings of these evoked potentials after the transection showed a definite lengthening of the period of time in which the primary cortical photic potentials would attain habituation. This lengthening was even more striking in the secondary cortical photic potentials, since normally they reach habituation in a very short time.

To explain these results it was suggested (Palestini & Lifschitz, 1959) that an inhibitory influence, arising in structures situated below the level of the lesion, was being exerted. The present paper presents results, obtained in preparations with a mid-pontine pre-trigeminal section, on the changes of the photically evoked cortical potentials, which give additional support to this hypothesis.

METHODS

Fifty cats were used in these experiments. After anaesthesia with sodium pentobarbitone, extradural monopolar stainless-steel electrodes were implanted on the suprasylvian gyrus, on the gyrus lateralis and gyrus lateralis anterior.

Control recordings were taken from these animals, non-anaesthetized, at least 5 days after implantation. The light stimulus in all experiments consisted of flashes at a rate of one per second from a Grass PS 1 photic stimulator. To ensure relatively constant stimulation of the retina the light source was always held at the same distance from the eyes, the animal was slung in a hammock and head excursions were restrained by an aluminium cone. All cats were dark-adapted at the beginning of each experiment and their pupils dilated with homatropine. Satisfactory isolation from outside sounds was achieved in all experiments. As a further means of ensuring a constant stimulus to the retina, the nictitating membranes were removed and the facial nerves were divided in some animals.

Both monopolar (with an indifferent electrode on the frontal sinus) and bipolar electrodes were used and the electrical changes were recorded by a Grass e.e.g. machine on paper or photographed from an oscilloscope trace. In the latter method successive cortical responses were superimposed on the same picture.

After securing control recordings of approximately one hour's duration daily, on at least 3 consecutive days, a pre-trigeminal transection, either mid- or rostropontine, was made under ether anaesthesia according to the procedure described by Batini, Moruzzi, Palestini, Rossi & Zanchetti (1959). The plane of the section is shown in Fig. 1. Recording was re-started 6–12 hr after the operation under the same conditions as for normal preparations and repeated daily for as long as they remained in good condition, usually not longer than 4 days. After the last recording the animals were killed and examined for cerebral oedema and any other abnormality.

In order to check the level and completeness of the brain-stem section the brains were fixed in 20% formalin, cut in the horizontal plane and stained by the Nissl and Weil techniques.

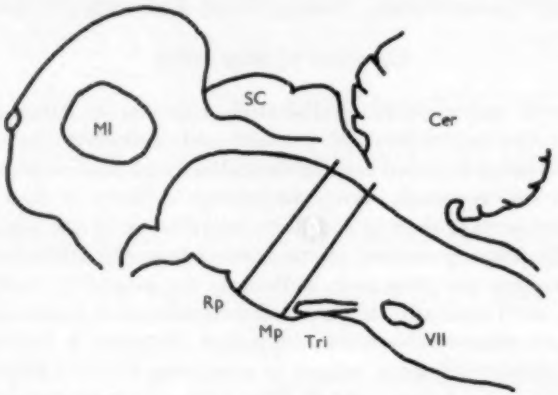

Fig. 1. Diagram of a longitudinal section of the brain stem of the cat, showing the position of the transections in relation to the pons and trigeminal nerve roots. Rp, rostropontine transection; Mp, mid-pontine transection; Cer, cerebellum; MI, massa intermedia; SC, superior colliculus; Tri, roots of trigeminal nerve; VII, roots of facial nerve.

RESULTS

Changes in the secondary cortical photic responses after pre-trigeminal transection

In normal cats a brief flash stimulus elicits the so called secondary photic response from the suprasylvian and anterior lateral gyri. Buser & Borenstein (1959) have shown that it differs distinctly from the primary responses recorded from the visual cortical area. In the normal awake cat the secondary response is of small amplitude and negative potential, with longer duration than that of the primary response. Its latency, about 20 msec, is variable and hence very difficult to measure in pictures of superimposed records (Fig. 2). To observe this secondary response more clearly it is necessary to start recording from the beginning of repetitive stimulation, and to superimpose several responses on the film, because at a stimulation rate of one flash per second the amplitude of the

secondary response decreases rapidly and is soon masked by spontaneous cortical activity.

After a pre-trigeminal mid-pontine brain-stem section, several changes are apparent in this secondary photic response:

(1) The amplitude increases, frequently reaching double that of normal cats, so that it stands out clearly against the typical desynchronized cortical activity of the mid-pontine pre-trigeminal preparation (Fig. 2).

(2) The latency remains constant at about 20 msec.

(3) In some cases the negative potential is preceded by a positive phase, so that the secondary response resembles that observed by Buser & Borenstein (1959) on cats anaesthetized with chloralose. In our experiments, however, the background cortical activity remained desynchronized.

(4) The amplitude of the secondary response decreases only after continued repetitive stimulation for several hours (Palestini *et al.* 1959).

Fig. 2. Potentiation of the secondary photic cortical potential after mid-pontine pre-trigeminal transection; oscilloscope recording. The dot in the diagram of the cortex shows the position of the monopolar recording electrode. *A–B*: single response (*A*) before and (*B*) after the transection. *C–D*: superimposed records showing the same changes (*C*) before and (*D*) after transection. Dots mark the stimulus artifact. Upward deflexion positive.

Two of these effects, the slow decrease in amplitude and the potentiation of the secondary response, appear also in cats with a rostropontine lesion, who usually show a synchronized cortical activity (Fig. 3).

Changes in the primary cortical photic responses after pre-trigeminal transection

The primary cortical photic potentials recorded at the gyrus lateralis in cats have been described by many investigators (Albe-Fessard, 1957). The typical response has five components. The first deflexion in our records had a mean latency of about 12 msec, and all five components were not always clearly visible. While there was some variability in the amplitude

of the different components this was minimized by photographic superimposed traces, and such records when measured for amplitude, as was done by Schoolman & Evart (1959), formed a more reliable basis for comparison.

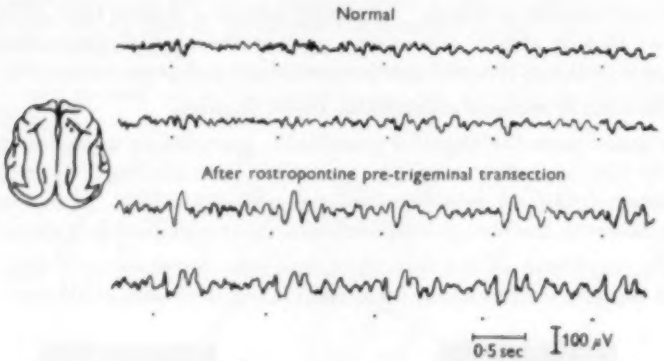

Fig. 3. E.e.g. showing synchronized background activity and potentiation of the secondary photic cortical potential after rostropontine pre-trigeminal transection. Dots on diagram at left indicate the position of the bipolar electrodes. Dots below records indicate the light stimulus.

A pre-trigeminal mid-pontine lesion produced changes in the primary cortical photic potentials (Figs. 4 and 5). A consistent increase in the amplitude of positive waves 3 and 4 and negative wave 5 was observed. Positive wave 1 remained almost unchanged.

After a rostropontine lesion this primary response showed a marked potentiation, though interference from large synchronous waves made its appearance sometimes irregular (Fig. 6). In a few instances after pre-trigeminal section the primary response showed a marked potentiation, even when the secondary response remained unchanged.

In order to check the possibility of interference from factors such as closure of the eyelids and/or the nictitating membrane, and pupillary changes, all the experiments of the foregoing type were performed in a few cats whose nictitating membranes had been removed, whose pupils had been dilated with homatropine and whose eyelids had been paralysed by nerve section. The results from these cats were the same as those described above.

DISCUSSION

The fact that the results described above are obtained consistently from a few hours after transection up to the end of the survival period (about 4 days in our experiments) makes it rather improbable that the potentiation

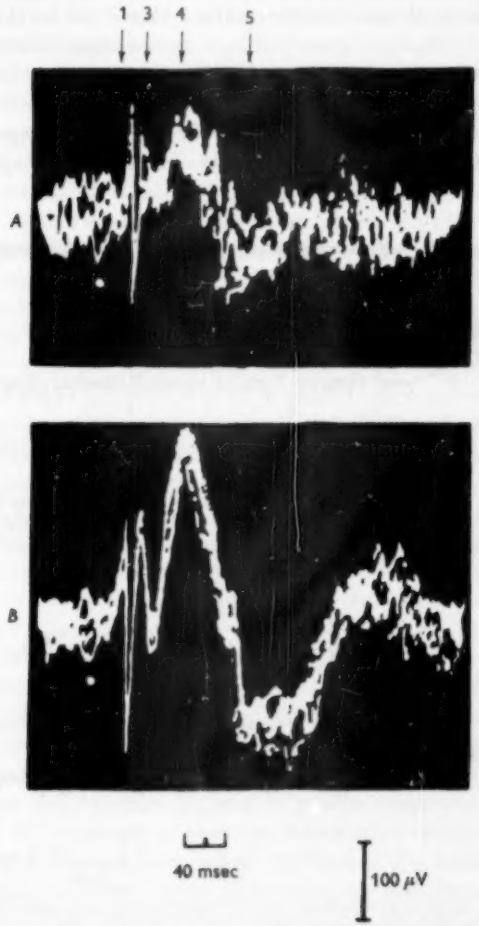

Fig. 4. Oscilloscope records from primary visual area to show potentiation of the primary photic cortical potential after mid-pontine pre-trigeminal transection. Deflexions numbered according to the nomenclature of Schoolman & Evart (1959). *A*: superimposed tracings of evoked potentials before the transection. *B*: superimposed tracings of evoked potentials after transection. Monopolar recording. Upward deflexion positive. Dots mark the position of the stimulus artifact.

of the cortical photic potentials, both primary and secondary, is attributable to an irritative stimulation from structures above the level of lesion. In the experimental situation described here such a stimulation would probably affect all the rostral nervous structures, especially the cortex, and would gradually decrease and finally vanish. Furthermore, such an irritative action might be expected to affect all the components of the primary cortical photic response equally, but in these experiments the

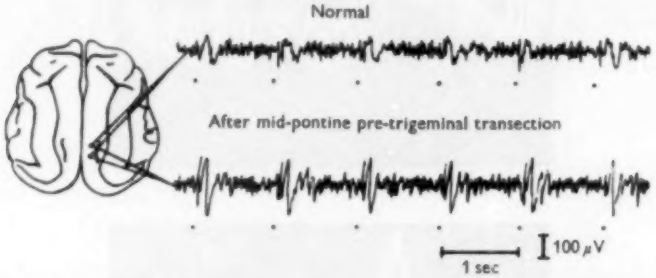

Fig. 5. E.e.g. showing desynchronized background activity and potentiation of the primary photic cortical potential after mid-pontine pre-trigeminal transection. Bipolar recording. Dots below records indicate the light stimulus.

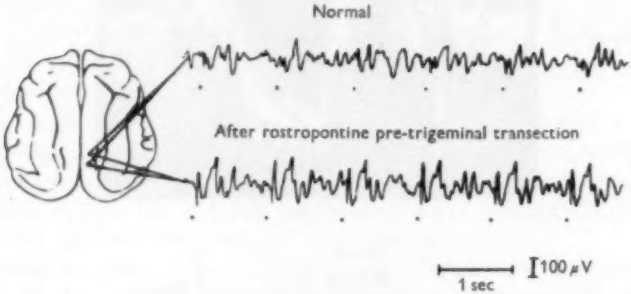

Fig. 6. E.e.g. showing synchronized background activity and potentiation of the primary photic cortical potentials after rostropontine pre-trigeminal transection. Bipolar recording. Dots below records indicate the light stimulus.

first component remained practically unchanged after the transection. If the irritative effect appeared only at the cortical level, both primary and secondary responses would be equally affected. However, it has been pointed out that in several cases there was a potentiation of primary responses while secondary responses remained unchanged. Furthermore, Palestini, Armengol, Mendoza & Lifschitz (unpublished results) have recently seen cats with a semi-pre-trigeminal transection that present

potentiation of the primary cortical photic response ipsilaterally only to the semisection of the brain stem, an observation obviously inconsistent with the irritative hypothesis.

Mid-pontine transection and secondary cortical photic potentials

Since Derbyshire, Rempel, Forbes & Lambert (1936) and Forbes & Morison (1939) first described a cortical response of long latency, elicited by an electrical stimulus applied to the sciatic nerve of a deeply anaesthetized cat, and Dempsey, Morison & Morison (1941), Dempsey & Morison (1942*a*, *b*) and Morison & Dempsey (1942) showed it to be conveyed in a secondary pathway, many papers have been published on investigations into this non-primary sensory response, and they have been reviewed recently by Buser (1957).

Such features as the distribution on the cortex, latencies and wave shapes suggest a relation between the secondary cortical photic potentials described in this paper and those recently studied by Buser & Borenstein (1959). Yet certain differences are apparent. According to Buser & Borenstein secondary responses in association areas from a curarized, non-anaesthetized cat depend on the background activity, the maximum irradiation of these potentials being obtained when there is a medium level of vigilance. Our observations on non-curarized, non-anaesthetized cats confirm their results but with the difference that the secondary responses are potentiated, following either a mid-pontine pre-trigeminal lesion, with its desynchronized e.e.g. pattern, or a rostropontine pre-trigeminal lesion, when the e.e.g. pattern is synchronized. These results suggest that although there is normally a correlation between the amplitude of secondary photic potentials and e.e.g. pattern, it is not a causal one, i.e. the potentiation described here is not due to the presence of low voltage fast activity.

The fact that potentiation appears only after transection of the brain stem leads to the assumption that the transection eliminates ascending tonic inhibiting influences from below the level of the lesion and possibly also leads to an increased action of facilitatory connexions from above the lesion level. This assumption is supported by the observations of Bremer & Stoupel (1959) which show that stimulation of the mesencephalic reticular formation facilitates the secondary response, although this effect was obtained only by concurrent electrical stimulation of the optic pathway.

In brief, we assume that inhibitory influences originate caudally to the lesion and act directly or indirectly upon the secondary cortical photic potentials recorded from the suprasylvian gyrus and from the anterior lateral gyrus.

Mid-pontine pre-trigeminal preparation and primary cortical photic potentials

The origin of the different components of the primary response evoked in the visual area has been studied by many investigators, but is still a controversial subject. It is, however, generally accepted that the first wave reflects the activity of the optic radiation and waves 3, 4 and 5 have an intracortical origin (Albe-Fessard, 1957). As to the second component, Bishop & Clare (1952, 1953) think it shows post-synaptic activity of Golgi II neurones. Bremer & Stoupel (1956) suggest it comes from presynaptic intracortical activity, and Malis & Kruger (1956) think it could originate in a second group of afferent fibres in the optic radiation. This paper, though not dealing specifically with this problem, seems to support the idea that waves 1 and 2 have a different origin from the others, as both remain unchanged after brain-stem transection.

We are inclined to attribute the potentiation of primary cortical photic potentials to a suppression of inhibitory influences coming from structures below the lesion level and to the enhanced facilitating action of the mesencephalic reticular formation. In support of this view some papers that describe the influence of the reticular formation upon evoked cortical potentials can be mentioned. While some authors have described a decrease in the amplitude of evoked cortical potentials during electrical stimulation of the mesencephalic reticular formation (Hernández-Peón, Scherrer & Velasco, 1956), the more recent work of Bremer & Stoupel (1959), Dumont & Dell (1960) and Steriade & Demetrescu (1960) has demonstrated an enhancement of this response under similar conditions when they are evoked by electrical shocks applied to the sensory pathways. The facilitation described by both Dumont & Dell (1960) and Bremer & Stoupel (1959) in their papers affects the same components of the primary cortical responses as are found potentiated after a pre-trigeminal lesion.

These results support the idea, already enunciated (Palestini *et al.* 1959), that there is no correlation between the facilitatory action of the cortical photic evoked potentials and the synchronizing-desynchronizing mechanisms, since the facilitating effect is observed both in the mid-pontine pre-trigeminal preparation (desynchronized e.e.g.) and the rostropontine pre-trigeminal preparation (synchronized e.e.g.). Dumont & Dell (1960) have also described a similar dissociation.

Published results and some inferences from the work described here suggest the probable pathways and the site of action of the inhibitory influence normally acting upon the secondary and primary cortical photic evoked potentials. Three probabilities are worth further consideration:
(*a*) The inhibitory influence arising below the lesion level might act

through a depression of the activity of the facilitatory mesencephalic reticular formation.

(*b*) The inhibitory influence might act directly on the cortical neurones themselves. The present experiments seem to point towards this mechanism, since the post-transection potentiation affects predominantly waves 3, 4 and 5 of the primary cortical photic response, which are generally considered to be of intracortical origin (Albe-Fessard, 1957).

(*c*) Another probability is an effect on the diffusely projecting thalamic nuclei, either directly or indirectly through the mesencephalic reticular formation. This probability is given considerable weight by virtue of the fact that long ascending axons originating both in the medulla and mesencephalic reticular formation (Brodal & Rossi, 1955) have been described and the facilitatory effect that these nuclei exert on the visual cortex is well known (Jasper & Ajmone-Marsan, 1952).

SUMMARY

1. Changes of the primary and secondary cortical photic potentials were studied in non-anaesthetized cats after mid-pontine and rostropontine total transections.

2. Both kinds of evoked potentials showed a clear potentiation after the mid-pontine pre-trigeminal transection. Positive deflexions 3 and 4 and negative deflexion 5 of the primary cortical photic potential were especially enhanced.

3. The rostropontine transection showed the same potentiation.

4. It is postulated that this potentiation would be due to the elimination of tonic inhibitory influences arising caudally to the lesion and probably also to the releasing of facilitatory activity in structures above it.

5. The lack of correlation between the mechanisms responsible for the e.e.g. pattern and the amplitude of the cortical photic evoked potentials is discussed.

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ELECTROPHYSIOLOGICAL INVESTIGATIONS ON RENSHAW CELLS

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When Renshaw cells are synaptically activated by antidromic volleys entering the spinal cord through ventral roots, the initial frequency of discharge is often over 1500/sec; and, after a few impulses at above 1000/sec, the frequency progressively declines until the terminal responses are 20 or more milliseconds apart (Renshaw, 1946; Eccles, Fatt & Koketsu, 1954; Eccles, Eccles & Fatt, 1956; Frank & Fuortes, 1956; Curtis & Eccles, 1958*a*, *b*). The initial high frequency certainly indicates that a very intense synaptic stimulation is evoking discharges immediately the cell has recovered from the absolute refractoriness following the previous discharge. It has been generally assumed that the later progressive decline in frequency of discharge results from a smooth continuous decline in the intensity of synaptic stimulation; nevertheless, there are experimental observations that suggest a discontinuity between an intense initial phase of synaptic stimulation and a subsequent low and prolonged activity. For example, the blocking drug, dihydro-β-erythroidine hydrobromide is very effective in suppressing all but the first two or three discharges, which persist with almost unchanged latency and frequency even after very large doses (Eccles, Fatt & Koketsu, 1954; Eccles *et al.* 1956; Curtis & Eccles, 1958*b*; Brooks & Wilson, 1959). A further indication of an intense initial synaptic excitatory action is provided by the lengthened interval that often disturbs the rhythm between the second and third discharges (Eccles, Fatt & Koketsu, 1954, Fig. 6G, H; Frank & Fuortes, 1956, Fig. 12B).

In the present investigation synaptic stimulation of Renshaw cells has been examined both by studying the effect of variation in intensity of the stimulus on the time course of the repetitive response, and by employing intracellular recording to give the time course of the synaptically induced

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depolarization. A further variant of the intensity of synaptic stimulation has been obtained by making use of the depression that follows a conditioning volley in the same presynaptic pathway to the Renshaw cell. A systematic examination has also been made of the synaptic activation of Renshaw cells by antidromic volleys from a large variety of muscle nerves.

METHODS

The general experimental procedures with micro-electrode recording from the lumbosacral cord of anaesthetized spinal cats were similar to those already described in publications from this laboratory (Eccles, Fatt, Landgren & Winsbury, 1954; Coombs, Eccles & Fatt, 1955). Renshaw cells were recorded from extracellularly, and also, with varying degrees of success, intracellularly. They were synaptically excited by antidromic volleys in motor axons, which were set up by stimulation either of ventral roots or of various muscle nerves. In the latter case the appropriate dorsal roots had been severed so as to eliminate synaptic activation either through interneuronal pathways or via axon collaterals of reflexly discharging motoneurones. Many muscle nerves were mounted on stimulating electrodes, and could be rapidly tested in turn by a rotary switch device (see Eccles, Eccles & Lundberg, 1957). The nomenclature of the various muscle nerves is given in the description of Table 1.

RESULTS

Time course of synaptic activation of Renshaw cells

Variations in the intensity of the synaptic stimulus. When the antidromic volley in the alpha fibres of the ventral root was progressively decreased in size, the intensity of excitation of Renshaw cells decreased stepwise, as revealed both by the frequency and by the duration of their discharge (Renshaw, 1946; Eccles, Fatt & Koketsu, 1954; Frank & Fuortes, 1956). Renshaw cells also displayed several intensities of response when excited by maximal antidromic volleys in the alpha fibres of different muscle nerves (Renshaw, 1946; Eccles, Fatt & Koketsu, 1954). By graded submaximal stimulation of the muscle nerve evoking the most intense response of a Renshaw cell that cell could be made to respond in a manner closely resembling the responses evoked by maximal volleys in the less effective nerves. For example, Fig. 1A and G are virtually identical; as also Fig. 1B and H; and Fig. 1C and J, K, L. Thus it may be concluded that the responses evoked by antidromic volleys from different muscle nerves vary solely on account of the effectiveness of the respective synaptic excitatory actions, i.e. the variations have a purely quantitative basis. In parenthesis it may be noted that a similar conclusion has been reached for the monosynaptic activation of motoneurones for different afferent pathways, both homonymous and heteronymous (Eccles *et al.* 1957). It is therefore justifiable to employ both submaximal and maximal volleys from different muscle nerves (cf. Fig. 2A–E) when investigating the effects of variation in the amount of synaptic excitatory action on Renshaw cells.

When there was a smooth decline in the frequency of the repetitive response evoked by a maximal antidromic volley, as in Fig. 2E, progressive weakening of the synaptic excitation (E–A) caused, first, a shortening of the response and a more rapid decline of the frequency; but the first two or three discharges were much less affected until the stimulus was so weak that all later discharges had been eliminated (Fig. 2A, B). When the reciprocal of the response interval, i.e. the frequency, was

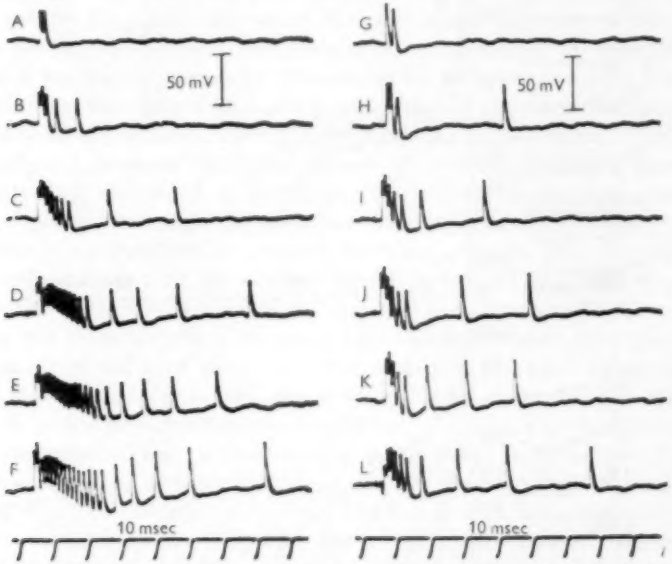


Fig. 1. Intracellular responses of a Renshaw cell evoked by submaximal alpha volleys from the medial gastrocnemius nerve, of increasing size from A–D and by maximal volleys in E, F. G–L are responses of same cell evoked by maximal alpha volleys from a series of other muscle nerves: plantaris, soleus, anterior biceps, inferior gluteal, lateral gastrocnemius and posterior biceps respectively. The series of A–E was taken a little later than F–L, the resting potential and spike potential having meanwhile declined. Same voltage and time scales throughout.

plotted for the successive discharges (Fig. 2G), it might be inferred for the range of responses of Fig. 2A–F that the synaptic stimulation diminished first in duration and only later in intensity. However, this inference is inadmissible because the initial very high frequencies of discharge (Fig. 2C–F) would be largely determined by the duration of Renshaw-cell refractoriness.

When a Renshaw cell was activated by various sizes of antidromic volley in the ventral root (Fig. 2H–L), the respective repetitive responses

may be plotted (Fig. 2M), as in Fig. 2G, to show the time course of the decline of frequency. Comparison of Fig. 2G and M shows that the two modes of grading synaptic excitation of Renshaw cells yielded closely parallel results, as has already been illustrated by the respective series of records, A–E and G–L in Fig. 1.

When attempting to derive the time course of the synaptic excitatory action from curves such as those of Fig. 2G and M, it may first be assumed

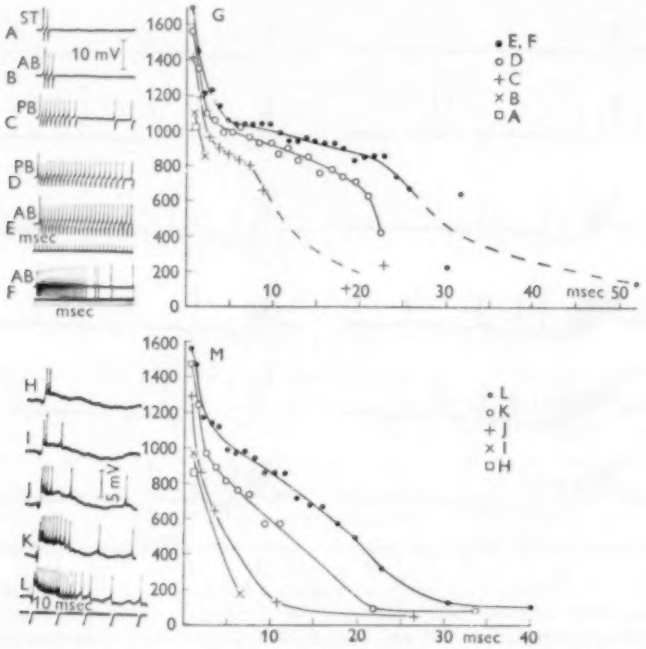
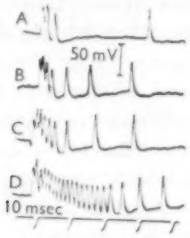


Fig. 2. A–F are responses of a Renshaw cell recorded extracellularly and evoked respectively by antidromic volleys from the three muscle nerves that were effective: maximal semitendinosus, submaximal anterior biceps, submaximal posterior biceps, maximal posterior biceps, and maximal anterior biceps at fast (E) and slower speed (F). The intervals between the successive responses are plotted as frequencies (ordinates) in G, much as has been done for interneuronal responses by Hunt & Kuno (1959), the abscissae being the time of each discharge after the initial discharge. Thus the curves of G (see symbol lettering) plot the time courses of the frequencies of discharge for each of the responses in A–F. The terminal portions of two curves are shown as interrupted lines because of the scatter of the points. H–L are another series for the repetitive responses of another Renshaw cell (with poor intracellular recording) evoked by an antidromic volley in L7 ventral root, which was maximal in L and with four grades of submaximal stimulation in H–K. The intervals between the successive spike discharges are plotted in M in the same way as in G, as again indicated by the symbol lettering.

that the various motor-axon collaterals converging on a Renshaw cell have similar time courses for their synaptic excitatory action. At least there has been no significant difference between the motor-axon collaterals for the motoneurones supplying different muscles, and no difference would be expected between axon collaterals from the lower-threshold and the higher-threshold alpha motor fibres. When there was a sufficiency of convergent impulses on a Renshaw cell, the initial intensity of activation was so high that the frequency (over 1500/sec) would be close to the limit set by the refractoriness of the cell; and even with considerably lower frequencies (around 1000/sec) refractoriness must be a significant factor in setting the frequency. The curves for the more powerful synaptic stimulations thus give a misleading impression of the time course for the intensity of the synaptic stimulus, because the refractoriness of the discharging cell prevents the initial intense phase from evoking a commensurately high frequency of discharge. The curves for the less powerful synaptic stimulations also give an erroneous time course, because a synaptic stimulus below threshold is given a zero value, as occurs after the first two or three responses with the weakest stimuli in Fig. 2G, M. However, some indication of the time course of the synaptic stimulus may be derived by noting the times at which the same response frequencies were produced by stimuli of different strengths. For example, the peak frequency in Fig. 2A was comparable with the frequency at a point 10 msec later in Fig. 2E, so it is probable that the respective intensities of synaptic stimulation were then similar. A complicating factor would be introduced by the possible action of accommodation in slowing the frequency in the latter part of Fig. 2E. Likewise, in the series of Fig. 2H–M, the peak frequency of the weakest response (Fig. 2H) was comparable with the frequency of the strongest response (L) at a point 10 msec later. An intermediate stimulus strength (J) showed a decline from an initial frequency of 1290/sec to 135/sec at 10 msec later; yet, with the response evoked by the maximum stimulus (L), the initial frequency of 1560/sec had declined only to 860/sec at 10 msec later. Evidently the synaptic stimulus was much greater in this latter case, but it could cause only a limited increase in the initial frequency. In general, therefore, the curves of Fig. 2G, M may be interpreted as indicating that the synaptic excitatory action produced by a single impulse had a very intense phase lasting 2 or 3 msec followed by a slowly declining tail of much lower intensity.

As already mentioned, an abnormally long interval may occur between the second and third responses to a very powerful synaptic stimulation. For example, in Fig. 3 this was regularly present in responses evoked by a maximum antidromic volley in the medial gastrocnemius nerve (D), but did not occur with the responses to the weaker stimulations provided by

other muscle nerves (A–C). The break in rhythm is further illustrated by the filled circles in the plotted curves (E). A regular observation has been that the lengthened interval is a little too brief (by about 0·2 msec in Fig. 3E) to be explicable merely as due to the dropping out of one response.



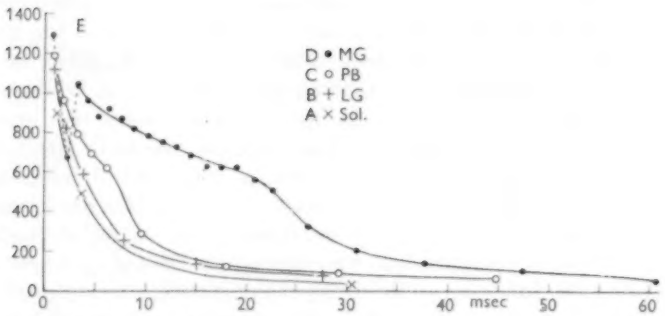


Fig. 3. A–D, repetitive Renshaw cell responses evoked by maximal antidromic volleys in the various muscle nerves as indicated. Same cell as in Fig. 1, with a faster sweep speed, in order to show the break in rhythm between the second and third responses in the largest response (D). In E the measurements from the series partly shown in A–D are plotted as in Fig. 2G, M. The plotted points for the medial and lateral gastrocnemius volleys are the means of two closely similar records. The curve joining the first three points for medial gastrocnemius (●) is drawn as a dotted line.

Similar findings occur with antidromic volleys set up by graded stimulation of the ventral root (Fig. 4A–D). The break in the rhythm was not present with the two weakest stimuli (A, B), but was very evident with the strongest stimulus (C), particularly when observed at high speed (D). Again, the lengthened interval was about 0·2 msec too brief to be attributable to the dropping of one discharge. Presumably, when extremely large the synaptic depolarization caused some disorganization of the spike-generating mechanism, just as occurs with cathodal depression of nerve

fibres or motoneurones (Coombs *et al.* 1955). Sometimes a small abortive spike was detectable at the time of the missing discharge (cf. Eccles, Fatt & Koketsu, 1954, Fig. 6G, H).

Another common type of disorganization of the spike mechanism is illustrated in Fig. 4E–H. Here the spike had a double composition resembling the compound IS–SD spike of motoneurones, and in the initial

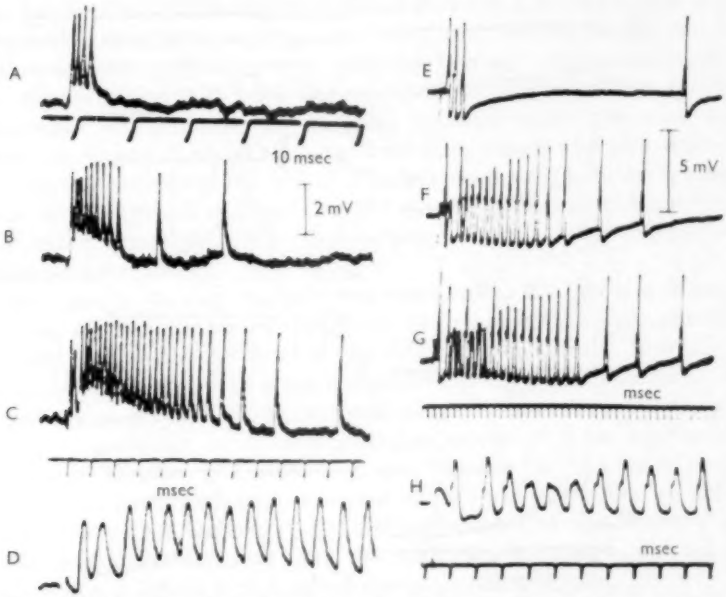


Fig. 4. A–D, series of Renshaw cell responses recorded intracellularly as in Fig. 3A–D, but for a different cell and evoked by antidromic volleys set up by stimulation of L7 ventral root, submaximal in A, B and maximal in C. D is same response as C, but at a faster sweep speed as shown above it. Same potential scale throughout. (D, H are at lower amplification.) E–H, responses evoked in another Renshaw cell as in A–D, G and H being evoked by maximal antidromic volleys in L7 ventral root. H at the sweep speed indicated below it.

high-frequency phase most of the spike potentials were largely composed of the initial small spike. In the fast record (H) it is seen that this initial small spike was fairly uniform throughout the response, while there was great variability in the later spike. Presumably this fragmentation of the spike is attributable to the high frequency of spike generation, rather than to the intensity of the background synaptic depolarization. It did not occur with the lowest frequency of discharge evoked by the weakest stimulation (Fig. 4E). On analogy with motoneurones it is probable that

the initial small spike was associated with the discharge of an impulse along the axon (Fuortes, Frank & Becker, 1957; Coombs, Curtis & Eccles, 1957*a*, *b*).

Excitatory post-synaptic potentials of Renshaw cells. On rare occasions the intracellularly recorded Renshaw cell responded by a depolarization

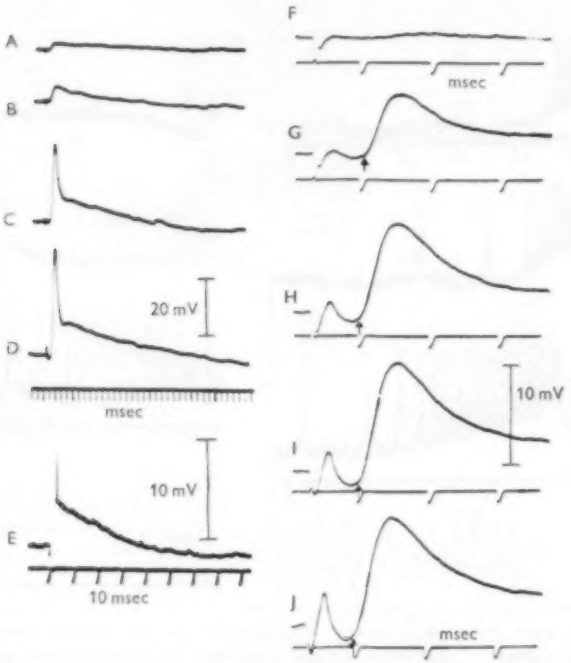


Fig. 5. Excitatory post-synaptic potentials (EPSPs) recorded intracellularly from a Renshaw cell with a recorded membrane potential initially of −60 mV. A–D show graded sizes of EPSPs evoked by submaximal (A–C) and maximal (D) antidromic volleys in L7 ventral root. E shows maximal response recorded at slower sweep speed and with a long time constant (1 sec) of the amplifier. F–J are fast records of EPSP evoked by progressively increasing antidromic volleys, as shown by the initial diphasic wave with first deflexion upwards. The arrows indicate times of onset of the EPSPs. Series E–J at higher amplification than A–D, as indicated. Upward deflexions signal positivity relative to the indifferent earth lead i.e. membrane depolarization.

(the excitatory post-synaptic potential, EPSP) uncomplicated by spike potentials. A response of this type has already been illustrated (Eccles, Fatt & Koketsu, 1954, Fig. 6I), but it was at that time suggested that the initial brief peak of depolarization was a spike potential. A much larger

potential of the same type is illustrated in Fig. 5. The responses of this Renshaw cell were so unusual that its identification could be regarded as established only after the full investigation described below.

The membrane potential was about −60 mV for the first records of Fig. 5; it gradually declined thereafter. A maximum alpha volley in the L7 ventral root evoked the large (36 mV) brief depolarization (D) that after about 2 msec merged into a much slower decaying depolarization. The full duration of this depolarization is shown in E to be about 60 msec. Progressive diminution of the alpha volley from maximum size (D) was associated with a diminution of both the fast and slow components of the depolarization (C). With the responses to smaller volleys the fast component was relatively lower, being but little above the slow component in B, and at about the same level in A. This is also well shown in the very fast records of Fig. 5, F–J. The fast component was barely identifiable in the weakest response (F), but showed progressive increment in G, H and I, there being no further increase when the alpha volley finally reached maximum (J).

The onset of the fast response was obscured in F–J, as it was superimposed on the downward (negative) deflexion of the extracellularly recorded soma spike potential of the alpha motoneurones. However, it can be detected as a sharp bend in the curve at the points marked by the arrows, which give it a latent period of about 0·4 msec from the phase of maximum positivity in the field potential produced by the approaching antidromic impulse. If a small allowance be made for the time of propagation of the antidromic impulse up the motor-axon collaterals, the actual synaptic delay for the onset of the depolarization of the Renshaw cell would correspond well with the value of about 0·3 msec calculated for other synaptic delays in the central nervous system (Eccles, 1957).

The smooth contour and relatively long duration (2 msec) of the brief depolarization establishes that it was not due to the superposition of a brief spike-like process on a more prolonged EPSP. This conclusion receives further support from the various gradations in its size (Fig. 5A–D, F–I). It may, therefore, be concluded that the potentials illustrated in Fig. 5 are EPSPs set up in a Renshaw cell by the summed action of several excitatory impulses converging on this cell. At least several of these impulses generated EPSPs having fast and slow components.

In Fig. 6A–H, from the same cell, a second maximum alpha volley was set up in the L7 ventral root at various intervals after the first. Even at relatively long intervals there was a considerable diminution both of the fast and slow components of the second EPSP. As shown by the plotted points (Fig. 6J) derived from the series partly illustrated in A–H, full recovery took about 150 msec, and at the shortest intervals (0·7–5 msec)

the depression was to about 40% of the control. As measured by the addition to the slow EPSP produced by the testing volley, the slow component of the EPSP was even more depressed at the shortest intervals. The time course of recovery was approximately the same for both the fast

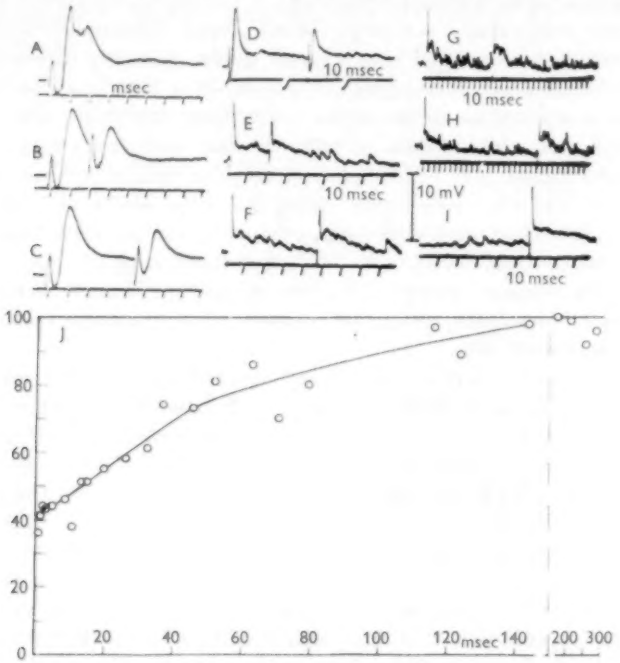


Fig. 6. A–I, same Renshaw cell as in Fig. 5, but responses evoked by two maximal antidromic volleys at various intervals as indicated by the stimulus artifacts. Note changes in sweep speed as indicated by the time bases for each record. Same potential scale throughout. J. Sizes of initial brief EPSP evoked by the second volley (calculated as percentages of the mean control size) are plotted against stimulus intervals for the series partly shown in A–I. Note the great compression of the time scale beyond the interrupted line at 150 msec.

and slow components. Renshaw (1946, Fig. 5) illustrated the depression of discharge that occurred with conditioning by a preceding antidromic volley in the same nerve fibres. There was considerable depression with a volley interval in excess of 50 msec, and more recently (Eccles, Fatt & Koketsu, 1954) it has been reported that the depression persisted for as long as 100 msec. Presumably this depression of Renshaw cell discharges is sufficiently explained by the depression of the second EPSPs illustrated in Fig. 6.

Patterns of activation of Renshaw cells

The input pattern from individual muscle nerves on to Renshaw cells has now been examined in conditions where nearly all the muscle nerves arising from the lower lumbar and upper sacral cord could be stimulated. The only nerves to hind-limb muscles which were not stimulated were the obturator nerve and sartorius nerve. The convergence on to Renshaw cells could thus be examined in almost full detail. Nine of the cells were dominated by one muscle nerve, which was at least twice as effective as any other muscle nerve in causing a discharge of impulses from the cell (Figs. 1F–L, 3A–D, 7A–D). All the remaining cells were excited really effectively by only 2 or 3 muscle nerves.

TABLE 1

The convergence of antidromic volleys in muscle nerves on to 18 Renshaw cells. Each muscle nerve was tested separately and in the columns are the numbers of impulses discharged from the respective Renshaw cell in response to a maximal antidromic volley in the alpha motor fibres. Numbers in bold type show the contribution from the dominant muscle nerves. The symbols labelling the columns correspond to the following muscle nerves, and the same convention is employed in the text: Q, quadriceps; SM, semimembranosus; SG, superior gluteal; FDL, flexor digitorum longus + flexor hallucis longus; Per., peroneal; Pl., plantaris; Pop., popliteus; LG, lateral gastrocnemius; AB, anterior biceps; ST, semitendinosus; IG, inferior gluteal; PB, posterior biceps; MG, medial gastrocnemius; Sol., soleus; T, posterior tibial nerve (flexor digitorum brevis plus medial and lateral plantar). Cells 10, 11 and 18 are illustrated in Figures 7, 2 and 1, respectively

Renshaw cell number	L6 and upper L7					Mid L7					Lower L7 and S1			
	Q	SM	SG	FDL	Per.	Pl.	Pop.	LG	AB	ST	IG	PB	MG	Sol.
1	6	5	**13**	.	.	.	.	.	.	.	.	.	.	.
2	.	.	**13**	.	2	.	.	.	.	.	.	.	.	.
3	.	.	**10**	.	5	.	.	.	.	.	.	.	.	.
4	.	.	**7**	2	.	.	.	.	.	.	7	.	.	.
5	.	3	**7**	3	**8**	.	.	.	.	.	.	.	.	.
6	.	.	**10**	3	3	.	.	3	**8**	3	3	.	.	.
7	.	**17**	4	.	8	.	5	.	.	.	.	.	.	.
8	.	**16**	.	.	8	.	.	.	.	.	3	.	.	.
9	.	.	.	.	.	**14**	9	9	.	.	.	1	.	.
10	.	.	.	.	.	6	.	**18**	.	.	.	.	8	6
11	.	.	.	.	.	.	.	.	**32**	2	.	**28**	.	.
12	.	.	.	.	.	.	.	.	**20**	9	.	**13**	.	.
13	.	.	.	.	.	.	.	.	.	**10**	.	**12**	1	.
14	.	.	.	.	.	.	.	.	.	.	5	**22**	.	.
15	.	.	.	.	.	3	.	6	.	5	7	**12**	9	.
16	.	.	.	.	.	.	.	**26**	.	.	.	.	**26**	**17**
17	.	.	.	.	.	.	.	5	2	.	5	4	**13**	.
18	.	.	.	.	.	2	.	8	7	.	7	10	**21**	4

Table 1 has been prepared so that the muscle nerves are arranged in the approximate sequence of their motoneuronal nuclei along the long axis of the ventral horn. When they are arranged in this way, an important feature of the input pattern to these 18 Renshaw cells becomes con-

spicuous; namely, that Renshaw cells tend to be excited by collaterals from the axons of adjacent motoneuronal nuclei, as for example in Fig. 7. When 2 or 3 muscle nerves were dominant, they were invariably from adjacent nuclei in the cord. An apparent exception to this rule (cell 4),

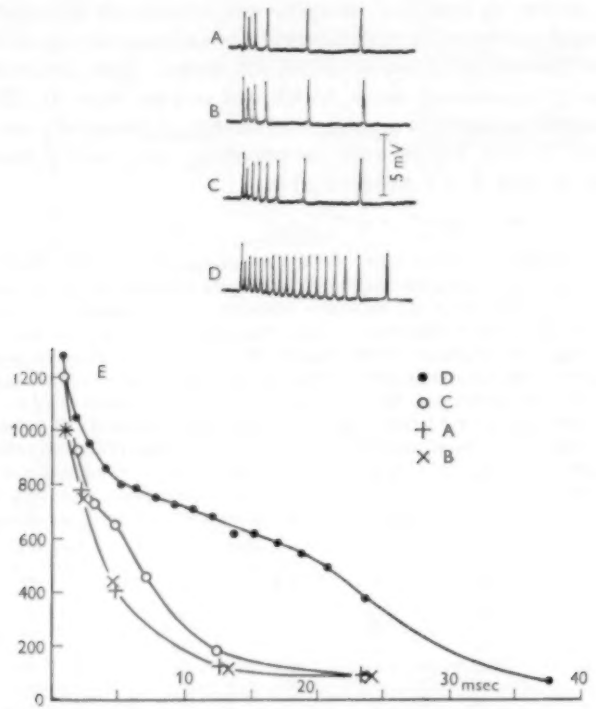


Fig. 7. A–D, Renshaw cell responses recorded intracellularly as in Fig. 3 and evoked by maximal antidromic volleys in the muscle nerves. A, plantaris; B, soleus; C, medial gastrocnemius; D, lateral gastrocnemius. All other antidromic volleys were ineffective. E, Responses illustrated in A–D are plotted as in Fig. 2G, H, the lettering of the symbol giving the reference to the records A–D. Note that the curves for A and B are virtually identical.

with equally effective inputs from superior and inferior gluteal nerves, can be accounted for by the imperfection of the tabular presentation. These two nuclei overlap in the ventral horn, and furthermore they are in the same cell column (Romanes, 1951). The influence of location was also apparent with the less effective muscle nerves. Thus cell 18 (Fig. 1) was excited most effectively by MG, but was also excited by PB, IG, AB,

Sol., Pop., LG and Pl. These nuclei are adjacent to MG, whereas Q, SM, SG and Per. were without effect and are more cephalad in the spinal cord. Motoneuronal nuclei in the same cell column in the ventral horn but separated longitudinally are less effective in exciting a Renshaw cell than are nuclei at the same level of the cord, but in different cell columns. Thus cells 7 and 8, which were fired optimally by SM, were not excited by AB, PB or ST, even though the latter are in the same cell column; whereas SG, Per. and Pop. were effective, yet lie in different cell columns in the ventral horn.

DISCUSSION

Synaptic excitation of Renshaw cells

The intracellular records of the EPSPs in Fig. 5 show a time course that corresponds closely with the time course that was postulated for the synaptic excitatory action generating the repetitive discharges of Figs. 1–4, 7. There is even direct evidence that submerged beneath the spike potentials of Figs. 1, 2H–L, 2A–D, 4A–D, there was an EPSP having a brief intense phase and a prolonged tail. For example, in the weakest response of Fig. 4A the four spikes were superposed on a depolarization that had a total duration of about 6 msec. With the next stronger response (B) the depolarization was about 10 msec in duration, but the second and third spikes were reduced in size, possibly owing to cathodal depression exerted by the large depolarization on which they were superposed. Finally, with the largest response (C) the spikes were superposed on a long tail of depolarization over 30 msec in duration, and between the second and third spikes appeared the break in rhythm that probably is further evidence of the initial intense phase of depolarization. The depolarizations of Fig. 4 were much less than those of Fig. 5, but the recording conditions were much inferior in Fig. 4, where the membrane potential and the spike potentials were only about -10 mV and 4 mV respectively.

The series of Fig. 2H–L similarly shows the spikes superposed on a prolonged depolarization up to 30 msec in duration, which is better seen when there were few spikes in H–J, than in L, for the depolarization was there largely submerged by the after-hyperpolarization that followed each spike. This after-hyperpolarization was very prominent in Figs. 1 and 4E–H. Evidently the generation of impulses had occurred at a locus remote from the site of recording.

It is surprising that the very large EPSPs of Figs. 5 and 6 failed to generate spikes. Comparable observations have recently been made with the cells of origin of the dorsal and ventral spino-cerebellar tracts (Eccles, Oscarsson & Willis, 1961; Eccles, Hubbard & Oscarsson, 1961), and with some cells of the dorsal horn (Eccles & Krnjević, 1959). It was suggested

that the spike-generating mechanism was suppressed by the depolarization resulting from injury by the micro-electrode. With motoneurones also the depolarization resulting from severe injury eliminates the production of spikes by the EPSP, there being a transitional phase of local responses. In all these examples the elimination of the spikes does not greatly affect the EPSPs. With motoneurones the time course is shortened as a consequence of the diminution of the membrane time constant (Eccles, 1961), the time course of the EPSP approaching nearer to that of the currents generating the EPSP. It may be concluded that the EPSPs of Figs. 5 and 6 give reliable information on the time course of the synaptic excitatory action that evoked the high-frequency discharges in other Renshaw cells. The high intensity of the brief initial synaptic excitation furthermore provides a sufficient explanation of the failure of large doses of dihydro-β-erythroidine to suppress the first one or two discharges (Eccles, Fatt & Koketsu, 1954; Eccles *et al.* 1956; Curtis & Eccles, 1958*b*; Brooks & Wilson, 1959).

Nevertheless, an important question still remains to be answered. It can be assumed that the EPSPs of Fig. 5 were generated by a virtually synchronous bombardment of the Renshaw cell by one impulse in each of several motor-axon collaterals. There is no evidence of any delayed bombardment via interneurones, which would be revealed by irregularities on the declining phase of the EPSP. Thus an explanation is required for the fast and slow components of the EPSPs produced by single impulses.

One suggestion would be that the initial rapid decline in the EPSPs of Fig. 5 is due to rapid destruction of acetylcholine by cholinesterase that is localized close to the site of its liberation. The subsequent low intensity of EPSP could then be attributed to the action of acetylcholine that had diffused beyond the zone of high cholinesterase activity. Unfortunately there are no observations of the effects produced by anticholinesterases on EPSPs such as those of Fig. 5. However, the action of anticholinesterases on the repetitive discharges of Renshaw cells indicates that the initial intense phase of action is not changed (Eccles, Fatt & Koketsu, 1954; Eccles *et al.* 1956), the only significant action being the intensification and great prolongation of the later low frequency component of the response.

Possibly, therefore, diffusion accounts for the initial rapid decline in intensity of action of the synaptic transmitter. However, on account of the electric time constant of the cell membrane, the rate of decline of the EPSPs of Fig. 5G–J will be much slower than that of the transmitter (cf. Curtis & Eccles, 1959). It may be assumed that diffusion out of the synaptic cleft would cause the transmitter concentration in the synaptic cleft to decline much faster than the EPSPs of Fig. 5G–J (cf. Eccles &

Jaeger, 1958, Fig. 3B). The prolonged residuum of action could then be due to the more diffuse action of acetylcholine on the membrane receptors adjacent to synapses. It has recently been shown (Miledi, 1960) that such receptors normally exist in an extensive zone around the region of the neuromuscular junction.

In the slower records of Fig. 6 there is clear evidence of brief depolarizing potentials occurring in random fashion. Since they have a time course rather like EPSPs, they may be assumed to be examples of the synaptic noise that has been reported on motoneurones and attributed to background internuncial bombardment (Brock, Coombs, & Eccles, 1952). In the anaesthetized preparation in which the activity of Fig. 6 was recorded, repetitive discharges of impulses from motoneurones and so up to motor-axon collaterals are unlikely to be the cause of all the activity seen in Fig. 6E–H. At least seven of these potentials occurred in Fig. 6I before the antidromic stimulus, hence the random potentials of Figs. 6E–H were not generated by the antidromic stimulation. However, there are synaptic connexions from interneurones to Renshaw cells (Curtis, Phillis & Watkins, 1961) and discharges from such interneurones could cause the spontaneous activity in Fig. 6. Alternatively, the small potentials could be comparable to the miniature end-plate potentials first described by Fatt & Katz (1952), and shown by them to be due to the liberation of quanta of transmitter. It should be remembered that the transmitter is the same as at the neuromuscular junction, and that the axon collaterals making synaptic connexions with Renshaw cells are indeed branches of the same motor nerve fibres that produce the miniature end-plate potentials of muscle. Possibly such miniature synaptic potentials are responsible for the late irregular discharges seen in such records as Fig. 2J, K, L and for the spontaneous discharges that are given by many Renshaw cells (Eccles *et al.* 1956; Curtis & Eccles, 1958*a*).

Convergence on to Renshaw cells from different muscle nerves

The convergence of muscle nerves on to a Renshaw cell was investigated by testing in turn the responses of the cell to single maximum alpha volleys in the motor fibres of the various muscle nerves. Conceivably this technique would demonstrate an arbitrarily restricted field of motoneurones from which a Renshaw cell could be excited during normal activity, for in such activity the repetitive firing of motoneurones might lead to the recruitment of Renshaw cells that were not excited by single volleys. This possibility was tested in one experiment by investigating the effect of repetitive synaptic activation of Renshaw cells. If a single volley failed to excite a Renshaw cell, a succession of volleys was also ineffective. The convergence revealed by the technique used in the present

work will thus also be present during the repetitive firing of motoneurones during normal reflex activity.

Individual Renshaw cells in Table 1 tend to be dominated by one or two muscle nerves. When there was only one dominant line, the Renshaw cell might be labelled as belonging to that particular muscle. When there was more than one dominant line, a convergence of synergists was sometimes evident, as in cells 13 and 16 in Table 1. Other Renshaw cells, however, were dominated by muscles of unrelated reflex functions, e.g. cells 11 and 12 were excited maximally by the hip extensor, AB, and the knee flexor, PB. The possibility of interaction between antagonists at the same joint is, however, limited by the topographical arrangement of the motoneuronal nuclei in the spinal cord. The knee extensors, Q, are more cephalad than the knee flexors, PB and ST, the centres of the respective nuclei being separated by more than one segment. This separation is sufficient to prevent any significant interaction between those antagonists. The ankle extensors, MG, LG and Sol., are more caudal than the ankle flexors (supplied by the deep peroneal nerve), but there is more overlap of the respective nuclei than with the knee antagonists. The location of motoneurones supplying the ankle antagonists thus again limits the possibility of convergence on to the same Renshaw cell, but there may also be other factors which limit the convergence.

SUMMARY

1. A detailed investigation has been made of the repetitive responses evoked in Renshaw cells by various sizes of antidromic volleys in alpha motor fibres. Comparable responses of a Renshaw cell were observed whether the size was varied by graded submaximal stimulation of a ventral root or by antidromic volleys in the different muscle nerves that converged on the same cell.

2. An analysis of the responses when there was a smooth decline in frequency from an initial maximum indicated that the synaptic stimulation had an initial very intense phase, about 2–3 msec in duration, followed by a much longer phase of low intensity. The very intense initial phase was also indicated by the break in rhythm sometimes observed between the second and third discharges, and by the high resistance of the initial 2 or 3 discharges to the depressant action of drugs such as dihydro-β-erythroidine hydrobromide.

3. An intense initial phase of synaptic depolarization was also revealed by intracellular recording from Renshaw cells. Usually the time course was obscured by superimposed spikes; but these spikes were occasionally absent and the full time course of the synaptically induced EPSP was revealed. There was an initial depolarization about 2 msec in duration

and up to 36 mV in height and a later slowly declining tail up to 60 msec in duration.

4. The EPSP evoked by a second antidromic volley was depressed for as long as 150 msec after a preceding volley, which correlates with previous reports of a prolonged depression of the discharges that a second antidromic volley evokes from Renshaw cells.

5. An investigation has been made of the pattern of activation of Renshaw cells from a large variety of muscle nerves, and its significance has been discussed.

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DISTRIBUTION OF RECURRENT INHIBITION AMONG MOTONEURONES

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In recurrent inhibition the discharge of impulses from motoneurones exerts an inhibitory influence not only on motoneurones of the same species (homonymous motoneurones), but also on other motoneurones of diverse function (Renshaw, 1941; Lloyd, 1946, 1951; Eccles, Fatt & Koketsu, 1954; Holmgren & Merton, 1954; Granit, Pascoe & Steg, 1957; Henatsch & Schulte, 1958; Kuno, 1959; Wilson, 1959; Brooks & Wilson, 1959; Wilson, Talbot & Diecke, 1960). It is now generally agreed that the recurrent inhibitory pathway runs via motor axon collaterals to special interneurones in the ventro-medial part of the ventral horn (Renshaw, 1946), which in turn discharge impulses that directly inhibit motoneurones (Eccles, Fatt & Koketsu, 1954; Frank & Fuortes, 1956; Eccles, Eccles, Iggo & Lundberg, 1961). Undoubtedly recurrent inhibition provides a negative feed-back whereby motoneuronal discharge causes inhibition that is extensively distributed to neighbouring motoneurones of diverse function. There is no evidence that recurrent inhibition extends more than one segment along the cord, and it is strictly ipsilateral.

In the earlier investigations the distribution of recurrent inhibition appeared to have no functional significance in reflex co-ordination and a general suppressor function on motoneuronal excitability was postulated (Renshaw, 1941; Eccles, Fatt & Koketsu, 1954; Holmgren & Merton, 1954; Hammond, Merton & Sutton, 1956). Subsequent investigators have attempted to discern additional functional meanings. Granit *et al.* (1957) found that recurrent inhibition was particularly exerted on tonic alpha motoneurones, which was confirmed by Kuno (1959), and proposed that recurrent inhibition served to stabilize the discharge of tonic motoneurones at low frequencies even during strong gamma excitation of muscle

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spindles (Granit & Rutledge, 1960). Brooks & Wilson (1959) also proposed a function related to the gamma-loop activation of motoneurones, but with a more general distribution to motoneurones of all types. When gamma motoneurones activate muscle spindles of a muscle, they evoke the discharge of Group Ia afferent impulses, which monosynaptically excite not only the motoneurones of that muscle, an homonymous action, but also the motoneurones of synergic muscles, and even of apparently unrelated muscles (Eccles, Eccles & Lundberg, 1957*a*; Eccles & Lundberg, 1958). These latter types of activation are usually weaker, and hence are more readily suppressed by the recurrent inhibition driven by the discharging motoneurones. Thus recurrent inhibition would tend to confine the operation of the gamma-loop mechanism to the alpha motoneurones belonging to the muscle containing the discharging annulo-spiral endings (cf. Granit & Rutledge, 1960), and hence subserve finesse of movement. Finally, Wilson, Talbot & Diecke (1960) investigated the distribution not only of recurrent inhibition, but also of the recurrent facilitation that was originally reported by Renshaw (1941) and later overlooked because it was depressed in anaesthetized preparations (Wilson, 1959; Wilson & Talbot, 1960). On the basis of this additional information they proposed that, besides the inhibitory stabilization of level of excitation of motoneurones and the sharpening of effectiveness of monosynaptic excitation, the recurrent facilitation serves to enhance the level of excitation of flexor motoneurones, which otherwise would be dominated by the more powerfully activated extensors.

The present investigation has been undertaken in relation to these various postulated functions of recurrent inhibition, which are tested by a more extensive survey of the distribution of recurrent inhibition than has hitherto been attempted. Altogether over 400 motoneurones belonging to fourteen different muscles of the cat hind limb have been studied intracellularly in order to discover the incidence of recurrent inhibition in response to antidromic volleys in fourteen different muscle nerves. In the original survey there were only thirty-six motoneurones and eight types of antidromic volley (Eccles, Fatt & Koketsu, 1954). The only other intracellular survey was restricted to twenty-eight gastrocnemius-soleus motoneurones and three types of antidromic volley (Kuno, 1959). A preliminary report has been published (Eccles, Iggo & Ito, 1960).

METHODS

The ten cats used were lightly anaesthetized with pentobarbital sodium. The spinal cord was cut at the upper lumbar region (lumbar 1 or 2) and all the ipsilateral dorsal roots from sacral 3 to lumbar 5 were cut. Up to fifteen nerves supplying the muscles of the hind limb were dissected free in the leg and set up for electrical stimulation. All the motor nuclei innervating knee and ankle muscles lie in the lumbar 6 and 7 and sacral 1 segments and

almost all the limb-muscle nerves, including the inferior and superior gluteal nerves, arising from this part of the cord were tested. A full investigation of the Renshaw inhibition for lumbar 5 and 6 segments has not been attempted because of the technical difficulty of working with many of the muscle nerves originating from these segments.

The techniques for intracellular recording from motoneurones have already been published in detail (Eccles, Fatt, Landgren & Winsbury, 1954; Coombs, Eccles & Fatt, 1955*a*). Single micro-electrodes filled with either 0·6 M-K_2SO_4-agar, or occasionally 3 M-KCl, were used. With KCl-filled electrodes the inhibitory potentials were tested at different membrane potential levels to see whether the inhibitory potential was masked by a change in the inhibitory equilibrium potential due to the leakage of Cl^- ions from the electrode (Coombs, Eccles & Fatt, 1955*b*; Eccles, Eccles & Lundberg, 1957*b*). The time constant of the amplifier (500 msec) was sufficiently long for recording the recurrent or Renshaw inhibitory post-synaptic potentials (RIPSPs) without appreciable distortion. Invasion of a motoneurone by an antidromic impulse in its own axon was prevented by passing between the micro-electrode and earth a brief pulse (0·1–1·2 msec) of hyperpolarizing current (see Fuortes, Frank & Becker, 1957; Coombs, Curtis & Eccles, 1957). In this way it was possible to employ a maximum antidromic volley in the homonymous nerve without the complication of an after-hyperpolarization in the motoneurone under observation. While thus avoiding depression of the Renshaw IPSP on account of superposition on the after-hyperpolarization, there was, nevertheless, still some depression because the IPSP was superposed on, and hence occluded by, the hyperpolarization that decayed for many milliseconds after the blocking pulse. By comparing the IPSPs of Fig. 1 *R* and *T*, the occlusion is seen to diminish the Renshaw IPSP to about 60 %. Throughout this paper there has been no correction for this effect, hence all homonymous IPSPs will be underestimated.

Nomenclature. The following abbreviations have been used, especially in figures: AB = anterior biceps; SM = semimembranosus; ST = semitendinosus; PB = posterior biceps; MG = medial gastrocnemius; LG = lateral gastrocnemius; Sol = soleus; Pop = popliteus; Pl = plantaris; FDL = flexor digitorum longus + flexor hallucis longus; Per = peroneal, supplying all three peroneal muscles as well as tibialis anticus, extensor digitorum longus and brevis; SG = superior gluteal; IG = inferior gluteal; T = tibial, supplying flexor digitorum brevis and the small foot muscles; Q = quadriceps; Grac = gracilis.

RESULTS

Post-synaptic potentials generated by recurrent inhibition (RIPSP)

As illustrated in Figs. 1, 2 and 3, maximum antidromic volleys in the motor fibres of many muscle nerves produce RIPSPs in a motoneurone. These diverse RIPSPs vary greatly in size, but have similar time courses: a central latency of rather more than 1 msec; a maximum at about 5 msec later; and a total duration of about 40 msec (cf. Eccles, Fatt & Koketsu, 1954). Since the equilibrium potential for the RIPSP (about -80 mV) is only about -10 mV from the resting potential (Coombs *et al.* 1955*b*), a linear increase in the inhibitory synaptic action will give a progressively diminishing increase in the size of the RIPSP, i.e. there is an interaction, or occlusion, between superimposed RIPSPs, which increases with the sizes of the RIPSPs (Fig. 3). Usually, the RIPSPs generated by single muscle volleys have not been larger than -2 or -3 mV, so there is no serious error in employing the amplitudes of the RIPSPs in Figs. 1 and 2

as measures of the respective inhibitory synaptic actions without making any correction for occlusion.

A total of 474 motoneurones were collected in the present experiments. As judged by the magnitude and instability of the resting and action potentials, many of the motoneurones were damaged by the penetration of the micro-electrode, and consequently were discarded as unreliable when assembling the statistical analyses in Tables 1 and 2. Because the sulphate-filled electrodes tended to be noisy and to block frequently, the resting potential measurements were not considered to be sufficiently reliable to provide an index of the condition of the cell. Instead, the size of the antidromic spike potential has been used and all cells with a spike potential of less than 50 mV were rejected.

In order to compare the total amount of recurrent inhibition which

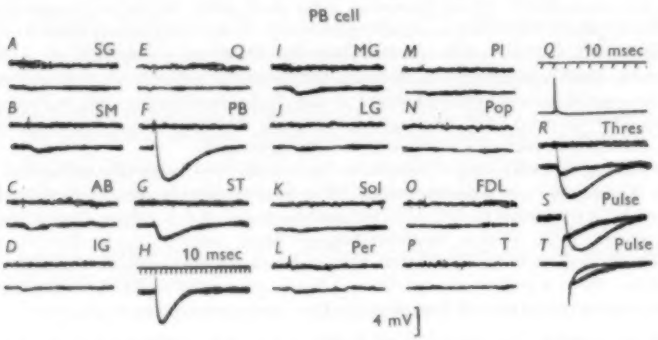

Fig. 1. Intracellular records from a posterior biceps motoneurone to illustrate the recurrent or Renshaw post-synaptic inhibitory potentials (RIPSPs) generated by antidromic volleys from different muscle nerves. Lower traces of *A*–*P* give the responses evoked by maximum antidromic volleys from the various muscle nerves as indicated by the symbols, which may be identified by reference to the paragraph, under Methods, labelled 'nomenclature'. Downward deflexion signals increasing negativity of the micro-electrode tip, i.e. hyperpolarization, *H* shows PB response at a slower sweep speed, all other records being at speed given in time scale of *Q*. Upper traces were taken from the dorsal surface of the cord. In *R* the RIPSP from *PB* was sometimes recorded since the stimulus was at the threshold of the axon itself. When the axon was activated the RIPSP was obscured by the larger after-hyperpolarization. In *S* a rectangular pulse was applied in about 50 % of the records, so preventing the invasion of the cell. In *T* the effect of such a pulse alone (upper record) is compared to the pulse plus the posterior biceps volley. The difference between them indicates the size of the autogenous RIPSP. Spike evoked by the PB volley was 89 mV (*Q*); conduction velocity of the axon was 70 m/sec; duration of after-hyperpolarization (AHP) was 100 msec (*H*); resting potential (RP) was −67 to −62 mV. Micro-electrode was filled with 0·6 M-K_2SO_4 plus 1 % agar. Same potential scale for records except *Q*. Records in this and subsequent figures consist of superposed sweeps.

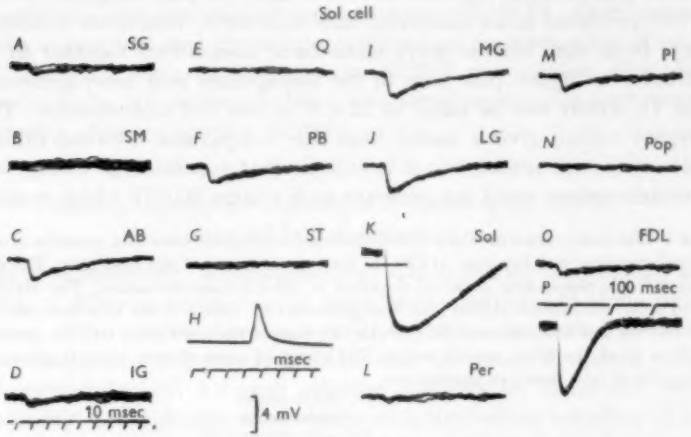

Fig. 2. Intracellular records as in Fig. 1, but from a soleus motoneurone in order to show the wide field of neurones from which it receives Renshaw inhibition. The size of the autogenous RIPSP is indicated by a line on the soleus record (*K*), where the large after-hyperpolarization follows the antidromic spike potential. This cell had a resting potential of −60 to −63 mV; the spike potential was 80 mV (*H*); the duration of the after-hyperpolarization was 140 msec (*P*); and the conduction velocity of its axon was 67 m/sec. Same potential scale for records except *H*, and time scale of *D* for all except *H* and *P*.

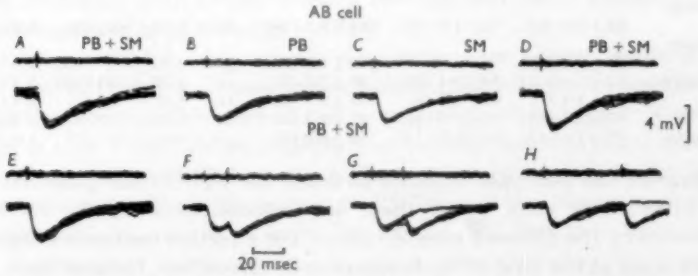

Fig. 3. These records from an anterior biceps motoneurone illustrate the RIPSPs produced by interaction of antidromic volleys from two different muscle nerves. In each record the upper trace is from a lead on the surface of the cord and the lower is the intracellular record. The RIPSPs were generated by maximal volleys in the alpha motor axons in posterior biceps (PB) and semimembranosus (SM) alone and together as indicated by the symbols; with synchronized volleys in *A*, *D* and *E* and with SM following PB at various intervals in *F*–*H*. At 50 msec there was almost no sign of the occlusion which was so evident in *E*–*G*. Antidromic spike (not shown) was 90 mV; autogenous RIPSP by pulse method was 3·2 mV; resting potential, −62 mV.

could be generated in different motoneurones, the peak amplitudes of the RIPSPs produced in an individual motoneurone by maximum antidromic volleys from each muscle nerve alone have been added together to give a cumulative figure (the sum of the autogenous and heterogeneous in Table 1), which was as large as 22 mV in one Sol motoneurone. These aggregate values give a useful basis for comparison between different species of motoneurones, but it is evident that synchronous volleys in all the muscle nerves could not generate such a large RIPSP which would be

TABLE 1. The mean values for the action potentials, conduction velocities, duration of after-hyperpolarizations and Renshaw IPSPs for the motoneurones of the hind limb. The nuclei are arranged in descending order of duration of after-hyperpolarization. The RIPSP is entered under autogenous RIPSP, i.e. that generated by volleys in the motoneurone's own muscle nerves and heterogeneous RIPSP, i.e. the sum of the individual RIPSPs generated by volleys in all the other muscle nerves. The standard error of each mean is given, with the number of cells tested in parentheses

Motoneuronal nucleus	Action potential (mV)	Conduction velocity (m/sec)	After-hyperpolarization: Duration (msec)	After-hyperpolarization: % of cells longer than 110 msec	Renshaw IPSP (autogenous) (mV)	Sum of all Renshaw IPSPs (heterogeneous) (mV)
Soleus	67 ± 3·2 (11)	64 ± 1·8 (12)	186 ± 3·5 (9)	100	1·46 ± 0·22 (7)	12·8 ± 1·6 (11)
FLD + FHL	66 ± 3 (15)	75 ± 2·1 (14)	118 ± 5·6 (13)	54	0·5 ± 0·25 (13)	3·4 ± 0·95 (16)
Plantaris	67 ± 2·3 (23)	75 ± 2·2 (15)	110 ± 4·9 (22)	45	1·16 ± 0·36 (9)	3·5 ± 0·65 (23)
Semimembranosus	72 ± 2·1 (10)	75 ± 5 (6)	110 ± 4 (10)	40	2·47 ± 0·12 (6)	6·6 ± 1·55 (6)
Anterior biceps	64 ± 1·8 (29)	76 ± 1·7 (31)	109 ± 3·9 (27)	37	2·06 ± 0·28 (17)	5·3 ± 0·36 (29)
Lateral gastrocnemius	66 ± 2·3 (24)	79 ± 2·1 (16)	105 ± 4·3 (19)	31	1·32 ± 0·11 (16)	4·1 ± 0·56 (24)
Medial gastrocnemius	62 ± 2·3 (32)	73 ± 1·1 (21)	103 ± 4·1 (25)	40	0·93 ± 0·2 (19)	2·9 ± 0·5 (30)
Gluteal	68 ± 2·3 (19)	—	103 ± 2·9 (18)	21	1·52 ± 0·2 (12)	2·9 ± 0·45 (19)
Posterior biceps	64 ± 1·6 (43)	79 ± 0·4 (46)	99 ± 2·5 (39)	10	1·52 ± 0·34 (29)	3·1 ± 0·49 (44)
Popliteus	69 ± 3·4 (7)	80 ± 2·9 (9)	98 ± 5 (6)	16	1·37 ± 0·25 (4)	3·8 ± 0·32 (7)
Peroneal	65 ± 2·1 (18)	85 ± 7·3 (4)	95 ± 2·5 (11)	18	0·45 ± 0·23 (8)	1·4 ± 0·42 (18)
Semitendinosus	66 ± 2·9 (13)	78 ± 0·5 (15)	89 ± 4·0 (9)	0	1·5 ± 0·25 (12)	1·9 ± 0·49 (13)

far beyond the potential required to reach the equilibrium potential for the IPSP. Not only would there be occlusion between the RIPSPs generated by the different muscle volleys, but a further occlusive influence would occur at the level of the Renshaw cells themselves, because there is a very effective convergence of the axon collaterals from the motor fibres to many different muscles on to the same Renshaw cell (Eccles, Fatt & Koketsu, 1954; Eccles *et al.* 1961).

With a few very sigificant exceptions the largest RIPSP was generated by an antidromic volley in the muscle nerve to which the impaled motoneurone belonged (Table 2, Fig. 9). The measurement of this autogenous RIPSP required that antidromic invasion of the impaled cell should be blocked by means of a short hyperpolarizing pulse through the recording

electrode as described in Methods. This autogenous RIPSP was largest with semimembranosus and AB motoneurones (Table 1). Exceptions to the rule were soleus and flexor longus digitorum motoneurones (Figs. 2, 9, 10; Table 2), where the autogenous RIPSP was smaller. The small number of alpha motor fibres in the soleus nerve presumably accounts for the poverty of its autogenous RIPSP.

Factors governing the intensity and pattern of the RIPSP received by a motoneurone

Type of motoneurone. Measurements of axonal conduction velocity and duration of after-hyperpolarization have been made on 146 alpha motoneurones. After pooling these motoneurones into eight categories according to conduction velocity, it is found that the mean durations of the after-hyperpolarization for each velocity category lie along the curve shown in Fig. 4. Evidently, those axons with conduction velocities of less than 70 m/sec come from motoneurones with relatively long after-hyperpolarizations. This confirmation of earlier investigations (Eccles, Eccles & Lundberg, 1958; Kuno, 1959) provides further support for the assumption that cells with long after-hyperpolarizations are tonic alpha motoneurones. If an arbitrary figure of 110 msec is taken to be the lower limit for the after-hyperpolarizations that are classed as belonging to tonic motoneurones, then the Sol sample in Table 1 exclusively comprised tonic motoneurones, whereas at the lower end of the scale ST was exclusively phasic. However, such a rigid criterion for tonic and phasic classification must be regarded as of doubtful validity.

Motoneurones with long after-hyperpolarizations always received a larger total RIPSP than motoneurones with brief after-hyperpolarizations, though the receptive field may be just as extensive. For example, a comparison of Figs. 1 and 2 shows typically that a soleus motoneurone received a larger aggregate RIPSP from much the same receptive field as for the PB motoneurone. Both the aggregate RIPSP and the duration of the after-hyperpolarization have been determined for 210 motoneurones. After pooling these motoneurones into eleven categories according to the duration of the after-hyperpolarization, the mean aggregate RIPSP was determined for each category and plotted as in Fig. 5. It is seen that there is an approximately linear relationship, the motoneurones with the longest after-hyperpolarizations having the largest aggregate RIPSPs. If an arbitrary figure of -9 mV is taken to indicate a very large aggregate RIPSP, then 75 % of the Sol motoneurones are in the group having very large RIPSPs. The SM comes next with 50 % of its cells in that same group followed by AB with 17 %.

could be generated in different motoneurones, the peak amplitudes of the RIPSPs produced in an individual motoneurone by maximum antidromic volleys from each muscle nerve alone have been added together to give a cumulative figure (the sum of the autogenous and heterogeneous in Table 1), which was as large as 22 mV in one Sol motoneurone. These aggregate values give a useful basis for comparison between different species of motoneurones, but it is evident that synchronous volleys in all the muscle nerves could not generate such a large RIPSP which would be

TABLE 1. The mean values for the action potentials, conduction velocities, duration of after-hyperpolarizations and Renshaw IPSPs for the motoneurones of the hind limb. The nuclei are arranged in descending order of duration of after-hyperpolarization. The RIPSP is entered under autogenous RIPSP, i.e. that generated by volleys in the motoneurone's own muscle nerves and heterogeneous RIPSP, i.e. the sum of the individual RIPSPs generated by volleys in all the other muscle nerves. The standard error of each mean is given, with the number of cells tested in parentheses

			After-hyperpolarization			
Motoneuronal nucleus	Action potential (mV)	Conduction velocity (m/sec)	Duration (msec)	% of cells longer than 110 msec	Renshaw IPSP (autogenous) (mV)	Sum of all Renshaw IPSPs (heterogeneous) (mV)
Soleus	67 ± 3·2 (11)	64 ± 1·8 (12)	186 ± 3·5 (9)	100	1·46 ± 0·22 (7)	12·8 ± 1·6 (11)
FLD + FHL	66 ± 3 (15)	75 ± 2·1 (14)	118 ± 5·6 (13)	54	0·5 ± 0·25 (13)	3·4 ± 0·95 (16)
Plantaris	67 ± 2·3 (23)	75 ± 2·2 (15)	110 ± 4·9 (22)	45	1·16 + 0·36 (9)	3·5 ± 0·65 (23)
Semimembranosus	72 ± 2·1 (10)	75 ± 5 (6)	110 ± 4 (10)	40	2·47 ± 0·12 (6)	6·6 ± 1·55 (6)
Anterior biceps	64 ± 1·8 (29)	76 ± 1·7 (31)	109 ± 3·9 (27)	37	2·06 ± 0·28 (17)	5·3 ± 0·36 (29)
Lateral gastrocnemius	66 ± 2·3 (24)	79 ± 2·1 (16)	105 ± 4·3 (19)	31	1·32 ± 0·11 (16)	4·1 ± 0·56 (24)
Medial gastrocnemius	62 ± 2·3 (32)	73 ± 1·1 (21)	103 ± 4·1 (25)	40	0·93 ± 0·2 (19)	2·9 ± 0·5 (30)
Gluteal	68 ± 2·3 (19)	—	103 ± 2·9 (18)	21	1·52 ± 0·2 (12)	2·9 ± 0·45 (19)
Posterior biceps	64 ± 1·6 (43)	79 ± 0·4 (46)	99 ± 2·5 (39)	10	1·52 ± 0·34 (29)	3·1 ± 0·49 (44)
Popliteus	69 ± 3·4 (7)	80 ± 2·9 (9)	98 ± 5 (6)	16	1·37 ± 0·25 (4)	3·8 ± 0·32 (7)
Peroneal	65 ± 2·1 (18)	85 ± 7·3 (4)	95 ± 2·5 (11)	18	0·45 ± 0·23 (8)	1·4 ± 0·42 (18)
Semitendinosus	66 ± 2·9 (13)	78 ± 0·5 (15)	89 ± 4·0 (9)	0	1·5 ± 0·25 (12)	1·9 ± 0·49 (13)

far beyond the potential required to reach the equilibrium potential for the IPSP. Not only would there be occlusion between the RIPSPs generated by the different muscle volleys, but a further occlusive influence would occur at the level of the Renshaw cells themselves, because there is a very effective convergence of the axon collaterals from the motor fibres to many different muscles on to the same Renshaw cell (Eccles, Fatt & Koketsu, 1954; Eccles *et al.* 1961).

With a few very sigificant exceptions the largest RIPSP was generated by an antidromic volley in the muscle nerve to which the impaled motoneurone belonged (Table 2, Fig. 9). The measurement of this autogenous RIPSP required that antidromic invasion of the impaled cell should be blocked by means of a short hyperpolarizing pulse through the recording

electrode as described in Methods. This autogenous RIPSP was largest with semimembranosus and AB motoneurones (Table 1). Exceptions to the rule were soleus and flexor longus digitorum motoneurones (Figs. 2, 9, 10; Table 2), where the autogenous RIPSP was smaller. The small number of alpha motor fibres in the soleus nerve presumably accounts for the poverty of its autogenous RIPSP.

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Type of motoneurone. Measurements of axonal conduction velocity and duration of after-hyperpolarization have been made on 146 alpha motoneurones. After pooling these motoneurones into eight categories according to conduction velocity, it is found that the mean durations of the after-hyperpolarization for each velocity category lie along the curve shown in Fig. 4. Evidently, those axons with conduction velocities of less than 70 m/sec come from motoneurones with relatively long after-hyperpolarizations. This confirmation of earlier investigations (Eccles, Eccles & Lundberg, 1958; Kuno, 1959) provides further support for the assumption that cells with long after-hyperpolarizations are tonic alpha motoneurones. If an arbitrary figure of 110 msec is taken to be the lower limit for the after-hyperpolarizations that are classed as belonging to tonic motoneurones, then the Sol sample in Table 1 exclusively comprised tonic motoneurones, whereas at the lower end of the scale ST was exclusively phasic. However, such a rigid criterion for tonic and phasic classification must be regarded as of doubtful validity.

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If the assumption that all alpha motoneurones with long after-hyperpolarizations are tonic is correct, the present results indicate that the tonic alpha motoneurones are more strongly inhibited by the recurrent collaterals than are the phasic motoneurones. This conclusion is in agreement with the results of the reflex experiments of Granit *et al.* (1957). There do not, however, appear to be two distinct populations of motoneurones;

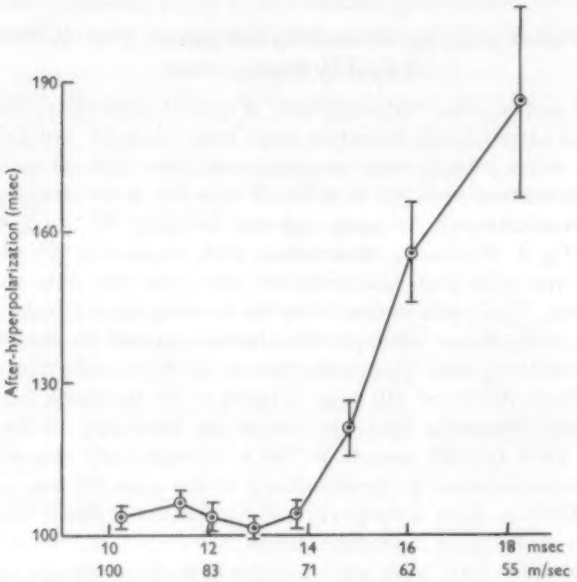

Fig. 4. Relationship between the conduction velocities of the axons and the durations of the after-hyperpolarization from 227 motoneurones. The times to travel 1 m are plotted as abcissae in linear scaling and the equivalent conduction velocities are also shown. On correction (assuming a $Q_{10} = 1{\cdot}7$) for the relatively low temperatures of the preparations (usually 34–35° C) the inflexion on the curve occurs at approximately 80 m/sec. The vertical lines indicate the sizes of the standard errors of the means at each point. Further description in text. The population was grouped into classes at intervals of 10 msec on the conduction velocity scale, except at either end where, because of the small sample available, the classes were larger.

all transitions exist both with the conduction velocities (Fig. 4) and with the after-hyperpolarizations (Fig. 5). The gamma motoneurones, on the other hand, do not develop a detectable RIPSP even though their axons are much more slowly conducting than those of the tonic alpha motoneurones (Granit *et al.* 1957; Eccles, Eccles, Iggo & Lundberg, 1960).

Location of the motoneurone. It was reported by Renshaw (1941) and by Eccles, Fatt & Koketsu (1954) that the effectiveness of recurrent inhibi-

tory action from muscle nerves on to a motoneurone was related to the proximity of this motoneurone to the motor nuclei of the various nerves. On the other hand, while recognizing the possible influence of proximity, Wilson *et al.* (1960) stressed particularly that a meaningful functional pattern could be discerned. Before attempting to relate the present results to the concepts developed by Wilson *et al.* three different procedures will be adopted in order to see how far the new evidence conforms to the

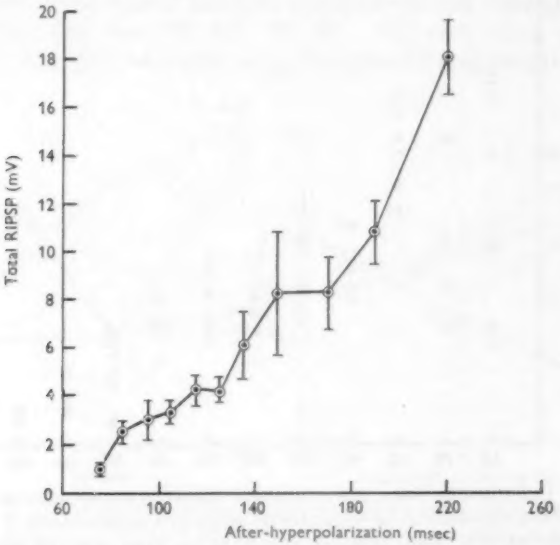

Fig. 5. Illustration of the relationship between after-hyperpolarizations of 210 motoneurones and the sums of all RIPSPs received by them from all the muscle nerves tested. Vertical lines indicate the sizes of the standard errors of the means. Further description in text. The population was arbitrarily grouped into classes at 10 msec intervals along the abscissa, except for the last four classes which were made larger to increase the size of the sample for statistical analysis.

proximity principle. In (i) and (ii) individual cells were tested whilst in (iii) populations of cells were examined.

(i) Figure 6 shows that when several cells in the same nucleus of one animal (AB in this example) were examined at the same segmental level, there was a fairly consistent pattern in the effectiveness of different muscle nerves in generating the RIPSP. The relative potency of these different nerves can be related in part to the proximity of the respective motor nuclei to the AB nucleus. For example, PB, SM and ST motoneurones lie in the same column of cells in the ventral horn and at overlapping segmental levels (Romanes, 1951), while the other potent nerves in Fig. 6

were MG and LG, which have their motoneurones just dorsal to the AB motoneurones, and IG with a just ventral location. These results for a single nucleus sampled at one level of the cord are illustrative of many series in the present investigation and are in general agreement with the proximity hypothesis.

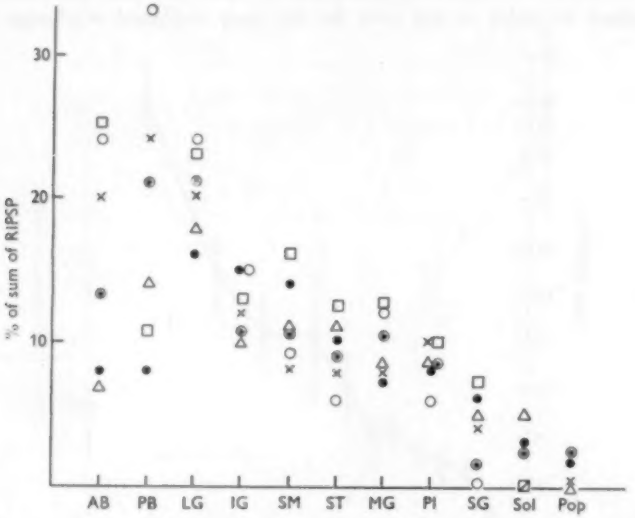

Fig. 6. In six anterior biceps motoneurones indicated by the six different symbols the RIPSPs received from all eleven nerves are plotted as percentages of the sums of all the RIPSPs. These neurones were all in the same region of the anterior biceps nucleus of one cat. The nerves are arranged approximately in descending order of effectiveness.

(ii) In an alternative procedure the RIPSP production has been determined for motoneurones in the same nucleus but at several different segmental levels. For this investigation the PB nucleus is particularly appropriate since it extends through nearly two segments. For example, the RIPSPs produced in PB cells by the most effective three muscle nerves were recorded at three segmental levels several millimetres apart, the mean values being plotted in Fig. 7*A*. The much greater effectiveness of the AB volley at the most rostral level (level I at lower L7) may be correlated with the more rostral location of its motor nucleus. This segmental gradient of effectiveness is even better illustrated in the larger sample of motoneurones plotted in Fig. 7*B*, where the mean RIPSPs recorded from all species of motoneurones were plotted as in Fig. 7*A*. The motor nuclei of PB, MG and IG correspond closely in their caudal extension, which is

several millimetres beyond the AB nucleus, as is indicated in the locations marked in Fig. 9. The segmental gradients of effectiveness of various muscle nerves in generating RIPSPs are thus in good agreement with the segmental locations of their respective motor nuclei.

(iii) There is also a dorso-ventral gradient in the patterns of RIPSP distribution. For example, Fig. 8 is formed by assembling the various motoneurones and their associated motor fibres into four groups that correspond to their dorso-ventral location, the dorso-ventral sequence being: FDL + Pl; MG, LG, Sol; PB, AB. ST; SG. For each group of motoneurones the RIPSPs are plotted as percentages of the aggregate RIPSP.

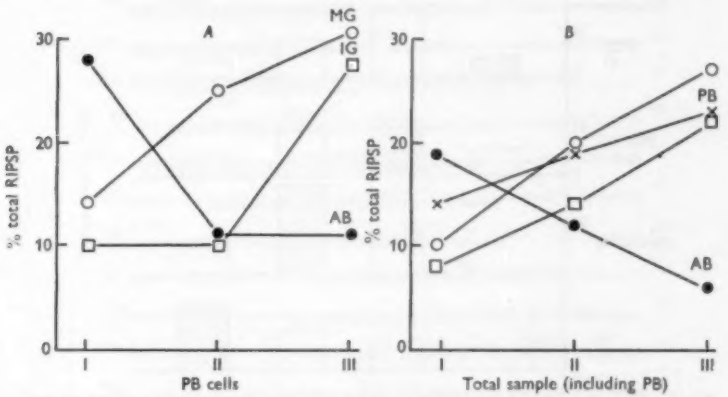

Fig. 7. *A*. In one cat posterior biceps motoneurones were investigated at different segmental levels: (I) in the lower L7 region; (II) 1 mm more caudally; and (III) in the upper S1 region. At each site the means of the RIPSPs from each source (MG, IG and AB) are expressed as percentages of the sum of all RIPSPs received. In *B*, mean RIPSPs in all the motoneurones in that same cat (including the posterior biceps that are alone plotted in *A*) are plotted for the three regions in the same way as in *A*, but RIPSPs produced by PB volleys are added. Crosses refer to PB volleys, otherwise symbols as in Fig. 7*A*.

There is seen to be a general tendency for the more ventrally located motoneurones to develop large RIPSPs in response to antidromic volleys to the corresponding motor nuclei, and in particular FDL and Pl volleys were quite ineffective on the two most ventral groups. Likewise the two most dorsal groups of motoneurones generate very little RIPSP in response to SG volleys. Neither FDL nor Pl, the most dorsal nuclei, give or receive RIPSPs of the same order of magnitude as more ventrally situated nuclei.

In order to compensate as far as possible for the incomplete sampling imposed by the experimental conditions, the results for all the experiments were pooled and the input pattern for each muscle nucleus was calculated.

The average value for the RIPSP from each muscle nerve to the average cell of the particular motoneuronal nucleus was then plotted on a histogram in Fig. 9. The importance of proximity is still evident. The peroneal nucleus at the rostral end of the series is not affected by the volleys in the muscle nerves of the more caudally located nuclei; reciprocally the motoneurones of the three most caudal nuclei (PB, MG and Sol) develop little if any RIPSP in response to volleys to the more rostrally located nuclei.

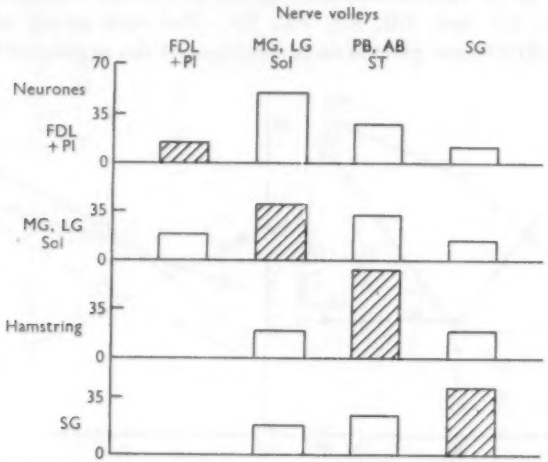

Fig. 8. The amounts of the RIPSPs from different sources are plotted as percentages of the sums of all the RIPSPs. The motor nuclei are arranged in horizontal rows (in relation to their dorso-ventral position): the most dorsal nuclei on the uppermost line; the most ventral on the lowermost. The corresponding nerve volleys are arranged in the vertical columns as indicated by the symbols. The height of each block is a measure of the sum of the RIPSPs which are produced by the antidromic volleys of its column, this sum being expressed as a percentage of the total RIPSPs of the motoneurones belonging to that row. With the hatched areas the RIPSP was produced in motoneurones by volleys of the same group.

The RIPSPs produced in Sol motoneurones provide an illustration of the patterns displayed in Fig. 9. The largest RIPSPs are produced by Pl, LG, AB, PB, MG volleys. The respective nuclei are either intermingled with Sol (LG and MG) or lie immediately dorsal (Pl) or ventral (AB, PB). The least effective volleys belong to nuclei more rostrally located (SM, SG, FDL, Per). An exceptional position is, however, occupied by ST which produces a very small RIPSP despite the proximity of the ST and Sol motor nuclei.

In order to display the pattern of distribution, the mean RIPSPs have been calculated for each type of antidromic volley acting on each type of

motoneurone. In Table 2 the volleys are arranged in columns and the motoneurones in rows. The antidromic volleys which were least effective were Per and FDL, which belong to the nuclei situated most dorso-laterally, and hence furthest from the location of most of the Renshaw cells (Eccles, Fatt & Koketsu, 1954). These motoneurones were also amongst the lowest recipients of RIPSP. Thus it seems that promixity

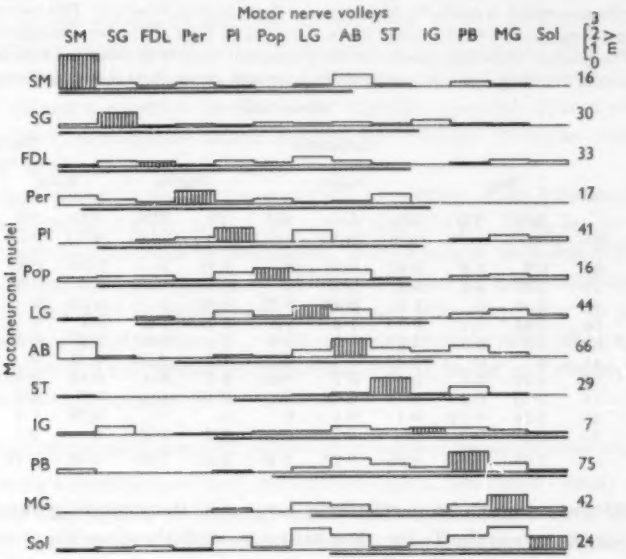

Fig. 9. The mean amounts of the RIPSPs received by all types of motoneurones are plotted for each type of antidromic volley in horizontal rows as indicated by the symbols. The motoneurones are arranged in vertical sequence on the left, the descending order representing relative rostro-caudal position. The RIPSPs produced by the various nerve volleys are arranged in vertical columns as indicated by the symbols above. Numbers to the right indicate the number of motoneurones in each category; the voltage scale applies to all potentials. The approximate longitudinal extent of each nucleus is indicated by the thick lines under each entry.

of the motoneuronal nuclei to the location of the Renshaw cells is an important factor in determining the size of the RIPSP. The poverty of action of Per volleys is presumably related to the extreme lateral course of their axons in the spinal cord (Balthasar, 1952), the motor axon collaterals consequently having a particularly long developmental path in order to reach the Renshaw cells.

Functional relationship of the motoneurones. In Table 2 the mean RIPSPs evoked by antidromic volleys in 387 motoneurones producing

extension and flexion at the various limb joints are tabulated with respect to functional relationship. Unfortunately the Table is incomplete: there were no hip flexors and the number of knee extensor (Q) motoneurones was so small that their RIPSPs were not included in the Table. The Q antidromic volleys were tested on all motoneurones, but uniformly had no action.

TABLE 2. The relation between functional groups of motoneuronal nuclei and the size of the RIPSPs generated in them by antidromic volleys in muscle nerves. The motoneurones producing extension and those producing flexion at hind-limb joints have been grouped and arranged vertically, while the muscle nerves stimulated have been arranged horizontally in the same order. Synergic groups are in bold figures and antagonists are in italic figures

		Nerve volleys										
		Extensors							Flexors			
		Hip		Ankle			Digits		Knee		Ankle	
Motoneurones	No.	AB (mV)	SM (mV)	MG (mV)	LG (mV)	Sol (mV)	Pl (mV)	FDL (mV)	PB (mV)	ST (mV)	Per (mV)	Total (mV)
AB	66	**1·9**	**1·4**	0·42	0·66	0	0·22	0	0·96	0·82	0	6·38
SM	16	**1·07**	**3·0**	0·2	0·2	0	0·1	0·2	0·5	0·2	0·4	5·87
MG	42	0·55	0	**1·3**	**0·68**	**0·23**	0·23	0	0·52	0	*0*	3·51
LG	44	0·84	0·12	**0·74**	**1·2**	**9·3**	0·71	0·2	0·4	0	*0*	4·51
Sol	24	1·3	0·1	**2·0**	**1·9**	**1·1**	1·1	0·37	1·2	0·22	*0·1*	9·39
Pl	41	0	0	0·52	1·0	0·3	**1·2**	**0·24**	0·23	0	*0·11*	3·9
FDL	33	0·37	0·1	0·44	0·7	0·32	**0·5**	**0·4**	0·16	0·12	*0·1*	3·21
PB	75	1·15	0·4	0·78	0·48	0·1	0·15	0	**1·7**	**0·6**	0	5·36
ST	29	0·20	0·12	0·1	0·1	0	0	0	**0·75**	**1·5**	0	2·77
Per	17	0·2	0·7	*0*	*0·1*	*0*	*0·2*	*0·2*	0	0·7	1·1	3·20
Totals		7·58	5·94	6·50	7·02	2·35	4·41	1·61	6·42	4·16	2·11	—

RIPSPs are seen to be particularly large within synergic groups, which are in bold figures in the Table. For example, with the synergic group of hip extensors the AB and SM volleys are particularly powerful on AB and SM motoneurones. Other examples are the ankle extensors (Sol, MG and LG), and the knee flexors (PB and ST). Evidently the large autogenous RIPSPs are just special examples of the large RIPSPs regularly occurring in the interaction between members of synergic groups. In all these examples the large RIPSPs could be attributable to proximity of the respective motoneuronal nuclei; there may be no significance in the functional relationship.

The RIPSPs produced in motoneurones by antidromic volleys from antagonist muscles at the same joint are shown in italic figures. These RIPSPs are always very small and are sometimes absent. It can be stated that RIPSPs between antagonist motoneurones are negligible. Again, however, this may not depend on their opposed functional relationship. In many cases there is a considerable distance between the antagonist motoneuronal nuclei. An exception would be the FDL, Pl and Per motor nuclei; the latter normally lies just lateral to the former two throughout almost the whole L7 segment.

Apart from the synergists at a particular joint, there is a tendency for the largest RIPSPs to be evoked by antidromic volleys from extensors on to extensor motoneurones. However, this relates to the most effective antidromic volleys, AB, MG and LG, rather than to all extensor antidromic volleys, and AB, MG and LG volleys are also effective on flexor motoneurones. Another relatively powerful antidromic volley is from the knee flexor, PB. On the other hand the knee extensor, Q, has little or no action on the extensors at other joints (Figs. 2, 10), an exception that is presumably attributable to the extensive segmental separation between Q motor nucleus in L5 and upper L6 segments, and the nuclei of all the other extensor muscles (in Table 2). Another general statement is that, apart from synergists, flexor antidromic volleys produce little or no RIPSP in flexor motoneurones.

Though there is much evidence that proximity of motoneuronal nuclei has an important influence on the size of the RIPSP, there is also evidence that other factors must be envisaged. For example, in Table 2 ST volleys are very poor at producing RIPSPs in Sol and G motoneurones. Reciprocally Sol and G antidromic volleys are also very poor in producing RIPSPs in ST motoneurones, yet the respective nuclei are in close apposition in the lower L7 and upper S1 segments, the ST being just ventral to the G and Sol motoneurones.

Excitatory post-synaptic potentials generated by antidromic volleys

In both anaesthetized and decerebrate cats, Renshaw (1941) regularly found a facilitation of monosynaptic reflex discharge when the conditioning antidromic volley entered the cord at a different segmental level from the tested muscle nucleus. Since Wilson (1959) has shown that the time course, as measured by changes in the size of the monosynaptic reflex, is longer for recurrent facilitation than for the inhibition, recurrent facilitation might be expected to be evident as a later depolarization. The latency of the facilitation was about 1 msec longer than for the inhibition, which Wilson (1959) suggested was due to at least one more interneurone in the facilitatory path.

Depolarization was never large in the present experiments though occasionally a small late depolarization was recorded (Fig. 10*K*). In order to see if a depolarization was being concealed by a larger hyperpolarization, the membrane was shifted to the equilibrium potential for the RIPSP (approximately -80 mV) by passing a steady current through the intracellular electrode and across the cell membrane, but no additional recurrent excitatory post-synaptic potentials (REPSPs) were revealed by this procedure. Except for one unusually large REPSP of 0·4 mV, all REPSPs were 0·1 mV or less. The total number of times that such a trace

as that of Fig. 10*K* was detected was 32 in a total of about 6000 trials. On 14 of these occasions a flexor motoneurone was depolarized, a peroneal motoneurone being involved ten times; which corresponds to the findings of Wilson, Talbot & Diecke (1960). The next most frequent examples were the extensor motoneurones, AB (8) and FDL (6).

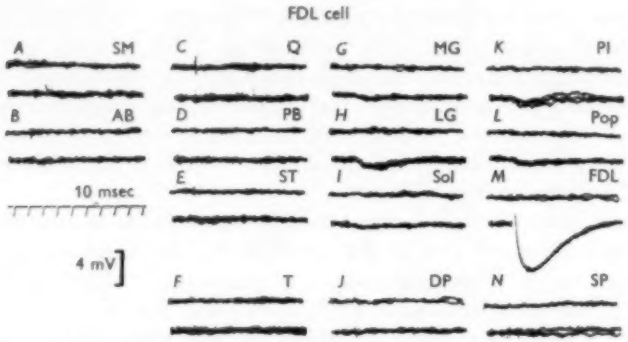

Fig. 10. The RIPSPs from all sources are shown for an FDL motoneurone as in Fig. 1. Note the small REPSP from plantaris (*K*). The antidromic spike was 58 mV; duration of the after-hyperpolarization was 110 msec and the resting potential was −54 mV.

DISCUSSION

In attempting to correlate the present results on the distribution of the RIPSP with the distributions reported by Wilson, Talbot & Diecke (1960), account must be taken of several differences between the two investigations. Recurrent facilitations were far more prominent in the investigation of Wilson *et al.* than were REPSPs in the present investigation, a difference readily explicable by the selective depression exerted by barbiturates on recurrent facilitation (Wilson & Talbot, 1960), although Renshaw (1946) observed large facilitations in cats anaesthetized with Nembutal. However, the present investigation has the advantage that detailed quantitative information is provided for many hundreds of motoneurones. Furthermore, the autogenous RIPSP was regularly measured, whereas autogenous recurrent inhibition was not investigated by Wilson, Talbot & Diecke (1960). Thus, the present investigation is much more comprehensive in respect of recurrent inhibition, but makes virtually no contribution on recurrent facilitation. It may be noted that there is on the whole very good agreement between Table 1 and the preliminary results of Eccles, Fatt & Koktesu (1954, Table 1) with relatively few motoneurones. The low values there reported for most autogenous RIPSPs arise because

in many cases they were submaximal, the stimuli being just below the threshold of the motoneurone's axon.

There is a very satisfactory degree of agreement between the present results on the distribution of RIPSP and those of Wilson, Talbot & Diecke (1960) on recurrent inhibition, particularly if allowance is made for the occasions when recurrent inhibition was masked by recurrent facilitation. For example, in agreement with Wilson, Talbot & Diecke (1960), there is in Table 2 virtually no RIPSP between the ankle extensors and flexors in either direction (G and Sol on the one hand and Per on the other); but there is a considerable RIPSP within each synergic group. There is similar agreement between the two series of investigations on the motoneurones responsible for extension and flexion of the digits, where likewise there is a negligible RIPSP between antagonistic motoneurones, which contrasts with the fairly large RIPSPs within each synergic group. Finally, there is agreement on additional interactions that occur between flexor and extensor motoneurones independently of functional grouping; G+Sol volleys produce an RIPSP in PB and ST motoneurones, which in Table 2 is seen also to be reciprocal; PB and ST volleys produce an RIPSP in AB motoneurones, which also is reciprocal in Table 2, at least for PB; finally Wilson, Talbot & Diecke (1960) reported that SM inhibits and AB facilitates Per motoneurones, though in Table 2 both actions were weakly inhibitory.

These last examples were reported by Wilson, Talbot & Diecke (1960) as being exceptions to their postulated general pattern of distribution of recurrent inhibition and facilitation. According to this pattern antidromic volleys from extensor muscles inhibit extensor motoneurones and facilitate flexors, while flexor antidromic volleys inhibit flexors and have little or no action on extensors. But in Table 2 there are several instances in which extensor volleys produce quite large RIPSPs in flexor motoneurones (AB → PG; G → BP; SM → Per); and similarly with flexors to extensors (PB → AB; ST → AB; PB → Sol). In fact, if the synergic groups be deleted from Table 2, there is on the average little more RIPSP from extensors to extensors (mean −0·35 mV) than from extensors to flexors (mean −0·28 mV) or flexors to extensors (mean −0·30 mV). Possibly, Wilson, Talbot & Diecke (1960) underestimated such types of recurrent inhibition because they were masked by recurrent facilitation. It can be concluded that the results documented in Table 2 provide many examples of distribution of RIPSP that do not conform with the postulates of Wilson, Talbot & Diecke (1960). In Table 2 there is no support for their proposal that the RIPSP has a distribution corresponding to that of Ib inhibition, which is very largely restricted to extensor motoneurones (Eccles *et al.* 1957*b*).

On the other hand, in Table 2 there is much support for the postulate that the distribution of RIPSP is related to the proximity of motoneuronal nuclei regardless of function. For example, AB and PB motoneurones are in the same neuronal column and have a considerable segmental overlap, but they have quite different functions, hip extension and knee flexion respectively. Yet in Table 2 there is a remarkable parallelism between the two motoneuronal types in respect both of action of the antidromic volleys, and of generation of RIPSPs by the motoneurones. However, there are several exceptions to the simple proximity hypothesis that was proposed by Eccles, Fatt & Koketsu (1954): ST motoneurones lie in a column just ventral to G and Sol motoneurones, and are at the same segmental level, yet there is virtually no interaction in either direction; the very poor interaction between the contiguous Per and FDL cell columns is another example. This latter case may arise on account of the lateral trajectory of the Per motor axons, as mentioned above, but the former case seems to require some functional discriminatory factor between flexor and extensor motoneurones in respect of RIPSP connexions.

The patterns of Renshaw cell connexions give an opportunity for investigating problems relating to the manner in which inhibitory connexions are established in development and to the possibility of changing the connexions in response to altered motoneurone function consequent on cross-union. Certainly the patterns are much less discriminative than with the Ia inhibitory action, which very largely operates between antagonists at a joint (Laporte & Lloyd, 1952; Hunt & Perl, 1960). Yet mere random growth and connexion seems inadequate to account for some of the observed specification of connexion or lack of connexion. Undoubtedly, a major factor governing the pattern of distribution is that the linkage by recurrent inhibition can be established only over short distances, regardless of the functional significance of the connexion if it could be established. The absence of inhibitory interconnexion between the motoneurones of knee flexors and extensors has been mentioned above. Instead there is strong recurrent facilitation in both directions (Wilson, Talbot & Diecke, 1960). This recurrent facilitation can operate over longer distances, which is presumably attributable to the one or more additional interneurones in the pathway (see Wilson, Talbot & Diecke, 1960; Wilson, Diecke & Talbot, 1960).

The present results agree with those of Kuno (1959) in fully confirming the important postulate of Granit *et al.* (1957) and Granit & Rutledge (1960) that tonic alpha motoneurones receive much more recurrent inhibition than phasic alpha motoneurones (cf. Holmgren & Merton, 1954). The tonic motoneurones were identified by the long duration of their after-hyperpolarization (Eccles *et al.* 1958; Kuno, 1959) and it was shown (Fig. 5)

that there was almost a linear relationship between the duration of after-hyperpolarization and the amount of RIPSP that a motoneurone received. On the basis of these two criteria it must be concluded that there are not two discrete categories of fast and slow motoneurones, but that all transitions exist (cf. Kuno, 1959). The specially large RIPSPs of motoneurones with long after-hyperpolarizations are further evidence that some specific factors control the development of recurrent inhibitory pathways in addition to mere proximity.

The general functional significance of recurrent inhibition is still an open question. Perhaps, as suggested by Wilson, Talbot & Diecke (1960), there are several functions. (i) There can be no doubt that the very wide distribution cutting across all functional classification must give recurrent inhibition a general suppressor action on motoneurones of diverse type, such as was originally suggested by Eccles, Fatt & Koketsu (1954). (ii) The high level of RIPSP distribution to tonic motoneurones would also act to stabilize the frequency of discharge during the maintenance of postures as proposed by Granit *et al.* (1957). (iii) Recurrent inhibition certainly would sharpen the operation of the gamma-loop activation of muscle as implied by Brooks & Wilson (1959); but it has yet to be demonstrated that such an action is functionally important in enhancing the precision of movement. (iv) Wilson, Talbot & Diecke (1960) incorporate recurrent facilitation in developing their concept that recurrent actions mediated through Renshaw cells tend to heighten the excitation of flexor motoneurones and depress the extensors, so helping to maintain a balance which otherwise would be weighted in favour of the more powerfully excited extensors. (v) Since tonic alpha motoneurones are special targets for recurrent inhibition, the intensive motoneuronal discharge subserving rapid movements would inhibit specifically the tonic motoneurones. This action would be functionally desirable, else the slowly contracting and relaxing muscles would impede the rapid movements. Thus recurrent inhibition would have the important function of suppressing all discharges from tonic motoneurones during the rapid movements of running or jumping. The desirability of this suppression was pointed out by Denny-Brown (1928) in his pioneer investigations on fast and slow muscles, and he observed suppression of discharges to soleus under such conditions.

There is experimental evidence for all these proposed actions of the recurrent pathways through Renshaw cells. It remains for further investigation to determine their relative importance in the control of posture and movement.

SUMMARY

1. The technique of intracellular recording has been employed in investigating the recurrent or Renshaw inhibitory post-synaptic potentials (RIPSPs) that are produced by a wide variety of antidromic motor volleys in the motoneurones of the seventh lumbar and first sacral regions of the cat spinal cord.

2. The total amount of the recurrent inhibition was measured as the aggregate of all the RIPSPs received by motoneurones of each type. It was much larger for motoneurones with long after-hyperpolarization, particularly for soleus motoneurones. For the whole population of motoneurones there was a significant relationship between the duration of the after-hyperpolarization and the size of the aggregate RIPSP.

3. The position of any motoneurone in the cord, whether in the rostral-caudal or in the dorsal-ventral dimensions, was related to the size and origin of the RIPSPs that it received. In general the closer the proximity of the motoneuronal nuclei the larger the size of the RIPSP that an antidromic volley in the axons of one produced in the motoneurones of the other.

4. Antidromic volleys in the nerves to flexor and extensor muscles exhibited approximately the same effectiveness in generating RIPSPs. Extensor motoneurones, however, received a larger aggregate RIPSP than did flexor motoneurones.

5. Occasionally an antidromic volley produced a small excitatory post-synaptic potential (the recurrent or Renshaw EPSP).

6. There is a general discussion of the various suggestions that have been made regarding the functional significance of the Renshaw or recurrent inhibition.

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THE DOUBLE TWITCH OF THE GRACILIS MUSCLE

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An interesting double contraction of the gracilis muscle of the cat, in which the first component had the unusually short contraction time of about 5 msec, was reported by Buller, Eccles & Eccles (1960). This very fast contraction contrasts with the contraction time of the remainder of the muscle, which is about 25 msec and thus equivalent to other fast muscles in the cat hind limb. This very fast initial contraction has no known parallel in other cat limb muscles and is comparable with the contraction time of the extra-ocular muscles. If it was produced by motor units which were independently innervated by alpha motor axons, it would provide a mechanism for making extremely quick movements.

In the present investigation we have prepared single motor units of gracilis muscle by stimulating ventral root filaments to see if there was a separation of gracilis muscle into fast and slowly contracting motor units. No such segregation was found, and the rest of the paper describes an attempt to analyse the mechanism of the double twitch.

METHODS

The animals (four cats) were anaesthetized with pentobarbital sodium. The gracilis muscle was carefully freed from the surrounding connective tissue without interruption of its blood or nerve supply. The remainder of the leg muscles were denervated. Either one thread was tied around its tendon of insertion (Fig. 1 G) or the tendon was divided into four parts (Figs. 2 and 3), each being independently secured to the strain gauge (Statham G 1-8-350 or G 1-80-35). The methods of recording twitches from muscles kept at a controlled temperature have already been described in detail (Buller *et al.* 1960).

Stimuli were applied, via an isolation transformer, to the whole or part of the L 6 ventral root for the gracilis muscle; or to L 7 ventral root for the lateral gastrocnemius muscle.

Single motor units of the gracilis muscle were obtained by teasing apart filaments of L 6 ventral root. It was often possible to dissect the filaments into a small portion containing only one active motor axon, which could then be stimulated.

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RESULTS

The isometric twitch of the gracilis muscle caused by stimulating electrically the L6 ventral root is shown in Fig. 1. Two components of the twitch are clearly seen, with the very fast component demarcated from the rest of the contraction by a step on the rising phase. The contraction times were 5–10 msec and 16–20 msec for the two components, and depended on the resting tension (Fig. 1). These twitches of the gracilis are very similar to those reported by Buller *et al.* (1960, Fig. 8). The relative amplitudes of the two components could be changed by altering the resting tension of the muscle. The first component was relatively larger at low tensions (*A* in Fig. 1) and increased more slowly in amplitude than the second component as the resting tension of the muscle was increased.

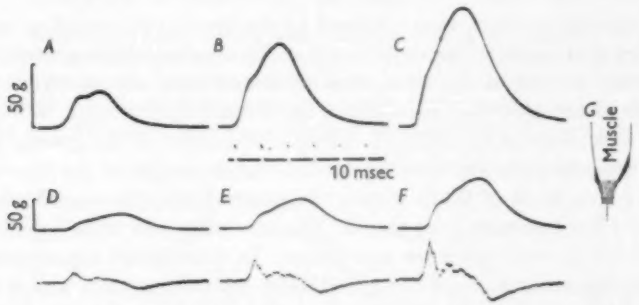

Fig. 1. Isometric twitches of the gracilis muscle are recorded in response to stimulation of L6 ventral root. *A–C* show the effect of increases in resting tension, the stimuli being maximal for all motor axons. *D–F* show the effect of progressively increased stimulus intensity, the resting tension remaining constant. The lower traces in *D–F* are differentiated records of the twitches which were recorded by one thread tied around the main aponeurosis of the muscle (see diagram). Time scale constant *A–F*. Tension scales for *A–C* and *D–F* respectively are given on the left. *G* illustrates the method of attaching the thread to the tendon.

When the stimulus intensity used to excite the ventral root was varied from threshold response to maximal response of the muscle, the two components of the twitch were present at all intensities (Fig. 1 *D–F*) and the first component was not proportionately larger at the low stimulus intensities. This result shows that the very fast component of the twitch was not due to muscle fibres innervated by alpha motor axons having specially low thresholds, which might be expected by extrapolation from the general relationships for alpha motor axons: the lower the threshold and the faster the conduction of the motor axons, the faster the contraction time of their motor units (Eccles, Eccles & Lundberg, 1958).

Twitches in single motor units of the muscle illustrated in Fig. 1 had contraction times for the peaks of the twitches similar to those for whole-muscle twitches. Each motor unit twitch also had some indication of the very fast component, usually as a convexity on the rising phase of the contraction. Twitch tensions ranged from 1·5 to 7 g (mean 5·7 ± S.E. 0·5 g, $n = 33$). The tetanus:twitch ratio ranged from 2·5 to 16, the lower the twitch tension the greater the ratio. For 8 motor units with twitch tensions less than 4 g the ratio was 9–16, whereas for the 4 motor units with twitch tensions of 10–14 g the ratio was only 2·5–5. The units with low ratios also had twitches with an earlier and more rapid rise of tension.

The above results suggested that the presumed very-rapidly-contracting muscle fibres in the gracilis muscle were not organized separately from the rest of the extrafusal muscle fibres, since, in two cats, none of the 42 single motor units had made only a very fast component in the twitch.

The records in Fig. 1 were obtained by the method of recording adopted by Buller *et al.* (1960). The aponeurosis of the muscle, which is inserted into the medial border of the tibia, was separated from the underlying aponeurosis of the sartorius muscle and the thread tied around it was connected to a strain gauge (Fig. 1). Close examination of the gracilis muscle during dissection showed that the curved ventral margin of the muscle gave rise to a thin sheet of fascia which was inserted into the superficial fascia overlying the gastrocnemius muscle. The two insertions of the gracilis were most obvious when the knee was flexed. In subsequent experiments the main aponeurosis was split longitudinally into three parts and a thread was tied to each. A fourth attachment to the muscle was prepared by tying a thread around the fascia inserted into the gastrocnemius (Figs. 2 and 3). With these four attachments to the muscle it was hoped to distribute more evenly the tension applied to the muscle during recording. All threads could be attached simultaneously or in various combinations, at approximately equal tensions, to the strain gauge.

With the new method of recording, the form of the twitch of the whole muscle depended on the part of the muscle from which the twitch was recorded. The smoothest rising phases, with no indication of a step, were recorded from the middle part of the muscle, *B* and *C* in Fig. 2. The very fast component was most conspicuous at the curved ventral border (Fig. 2*D*), i.e. when recording from the fascia which inserts into the gastrocnemius. There was also a smaller very fast component at the dorsal curved border of the muscle, Fig. 2*A*. The presence of the two parts of the twitch are most clearly revealed by the differentiated twitch records in Fig. 2.

The twitch was almost free from any initial step if the contracting fibres of single motor units were in the dorsal (rostral) half of the muscle; the

contraction was maximal at about 20–25 msec (Fig. 3*A*, *B*). When the active muscle fibres of a single motor unit were in the ventral (caudal) part of the muscle, a pronounced step at 8–10 msec was often present in the twitches (Fig. 3*G*). The contraction of a motor unit in the dorsal part of the muscle usually developed only a very small tension when it was recorded from the opposite edge of the muscle and vice versa; compare *A* and *B* with *C* and *D*, and *G* and *H* with *E* and *F* in Fig. 3. The step in the twitch,

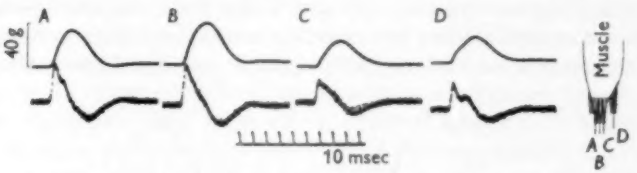

Fig. 2. Isometric twitches of the gracilis muscle recorded on stimulation of part of the L 6 ventral root. The twitches were recorded from each of the four attachments in turn, *A* being the rostral edge of the muscle and *D* the caudal. The diagram illustrates the method of recording from the muscle. Differentiation of the twitches, in the lower records, illustrates the existence of two components at the outer leads.

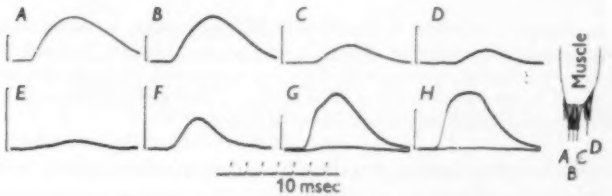

Fig. 3. Isometric twitches in 2 motor units from the gracilis muscle, recorded at each of the 4 leads from the muscle in turn (see diagram). The tension scales all indicate 10 g. The motor unit in *A–D* was at the rostral side of the muscle, and the unit in *E–H* was on the caudal edge. The tetanus:twitch ratios from *A–H* were respectively 5·4, 4·3, 7, 9, 7·4, 6·5, 4·2 and 4·3.

when present, was always most obvious in the part of the muscle which contained the active fibres as determined both by visual observation during the twitch and by the tendinous slip which gave the maximal tension. The maximal tensions for the two motor units in Fig. 3 were 18·5 and 16·1 g and were developed at leads 2 and 3 respectively. Visual inspection confirmed that the active fibres were in the dorsal part of the muscle for the upper unit and in the ventral part of the muscle for the lower unit. Although none of the 9 units was exclusively very-rapidly-contracting, some units had a large first component which was accentuated at low resting tensions.

The very fast component of the gracilis twitch was not, therefore, due to muscle fibres separately innervated by very-rapidly-conducting α motor axons. It was strongly influenced by the resting tension of the muscle and was most conspicuous when the contraction was recorded at the margins of the muscle where there is the greatest difficulty in obtaining uniform tension in the muscle fibres. These results suggested that the very fast component of the gracilis twitch was due not to the existence of special types of very fast muscle fibres, but rather to the non-uniform conditions in recording the contraction. In order to see if the very fast component could be produced in other fast skeletal muscles, an experiment was done with the plantaris and lateral gastrocnemius muscles. These were chosen

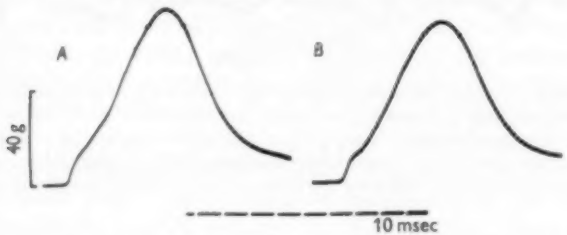

Fig. 4. Isometric twitches in lateral gastrocnemius muscle, recorded under low resting tension, to stimulation of the L 7 ventral root. In *A* the plantaris muscle, although not attached to the strain gauge, also contracted. In *B* the only contraction was in lateral gastrocnemius after section of the nerve to plantaris muscle. A very fast component is present in both *A* and *B*.

since they are closely united proximally and thus mimicked to some extent the conditions found in the gracilis muscle. The tensions on the two muscles could be changed independently, thus allowing the effect of unequal tension in the muscle mass to be analysed. A distinct very fast component, about 20 % of the peak amplitude of the twitch, was present when either the lateral gastrocnemius (Fig. 4*A*) or the plantaris muscle was under low resting tension. The step which marked off the very fast component at low tensions was still distinguishable as a small difference in slope at the start of the twitch at intermediate tensions. It was not detectable at higher resting tensions. This agrees with the results from the gracilis muscle in showing that the step arises in that part of the muscle under greatest tension but that it is conspicuous only when the muscle is at low resting tension. When the nerve to the plantaris was cut, so that only the lateral gastrocnemius contracted on stimulation of the L 7 ventral root, the initial step was at least as prominent as before (Fig. 4*B*).

DISCUSSION

The conclusion to be drawn from the present results is that the very fast component in the twitch is not peculiar to the gracilis muscle, although it may be particularly large in it under some conditions. The phenomenon is unlikely to have any physiological significance, since it has its origin in the method of recording and is most prominent in the flaccid muscle. It probably arises because the gracilis muscle is not uniform and different series compliances may exist. This may be expressed as a small part in the middle having a small series compliance compared to that of the mass of the muscle which lies on either side. Hill (1951) has demonstrated that an increase in the series compliance increased the contraction time (time to peak) in frog muscle. If it is assumed that this is applicable to mammalian muscle, it seems possible that the compound gracilis contraction is derived initially from those fibres with extremely small series compliance and the later part of the contraction originates from muscle fibres with a large series compliance in the recording system. This mechanism could account for the presence of an apparently very fast contracting component in the twitch when, in fact, all the muscle fibres had similar contraction times.

SUMMARY

1. The apparent double isometric twitch of the gracilis muscle was analysed by recording (*a*) from the whole muscle while stimulating the ventral roots and (*b*) from single motor units prepared by stimulating filaments dissected from the ventral roots.

2. The shape of the twitch depended on the method of recording and could also be varied by altering the resting tension on the muscle. The very fast component was most conspicuous and relatively largest at low tensions.

3. No motor units were found with a pure very fast contraction and a very quick component in the twitch was most evident in motor units at either edge of the muscle, particularly the caudal edge.

4. The very fast component, when present in motor units, was largest in those parts of the muscle where the tetanus:twitch ratio was least.

5. A double twitch could also be produced with both the plantaris and gastrocnemius muscles.

6. It was concluded that there were no separately organized very-rapidly-contracting muscle fibres in gracilis muscle.

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THE EFFECT OF CALCIUM AND MAGNESIUM ON THE SPONTANEOUS RELEASE OF TRANSMITTER FROM MAMMALIAN MOTOR NERVE ENDINGS

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In both amphibia and mammals the amount of transmitter released at the neuromuscular junction by a nerve impulse varies directly with the calcium concentration and inversely with the magnesium concentration in the surrounding medium (del Castillo & Stark, 1952; del Castillo & Engbaek, 1954; Boyd & Martin, 1956*b*). It might be expected therefore that changes in the concentration of these ions might affect in the same manner the spontaneous release of transmitter that is detected post-synaptically as miniature end-plate potentials (m.e.p.p.s). Previous investigations have shown that in mammalian preparations calcium ions do influence the m.e.p.p. frequency (Boyd & Martin, 1956*a*; Liley, 1956*d*), but in amphibian preparations no consistent effect has been detected (Fatt & Katz, 1952). Magnesium ions have not hitherto been found to change m.e.p.p. frequency in amphibia or mammals (del Castillo & Katz, 1954*a*; Boyd & Martin, 1956*a*).

The results of the present investigation differ from those of previous studies in that both calcium and magnesium have been found to affect m.e.p.p. frequency. This action is, however, exerted on only a fraction of the spontaneous release of transmitter, the remaining fraction being unaffected by the absence of calcium or the presence of magnesium in high concentration.

METHODS

All experiments were performed *in vitro*, with the rat diaphragm–phrenic-nerve preparation (Bülbring, 1946). The animals were male and female albinos of the Wistar strain weighing between 180 and 220 g. The method of dissection, the divided recording chamber and the mounting of the preparation have been previously described (Liley, 1956*a*). The phrenic nerve was immersed in paraffin oil, whereas the strip of diaphragm muscle was irrigated with a solution similar to that described by Liley (1956*a*). Its composition was (mM): NaCl 137·0, KCl 5·0, $CaCl_2$ 2·0, $MgCl_2$ 1·0, NaH_2PO_4 1·0, $NaHCO_3$ 12·0, Glucose 11·0. The experimental solutions resembled this solution except for the omissions or additions of the ion species under test. When the calcium concentration of the fluid was raised above 10 mM it was necessary to omit the NaH_2PO_4 and to reduce the $NaHCO_3$ concentration in order to

keep the calcium in solution. Additions of $MgCl_2$ and $CaCl_2$ were osmotically compensated for by withdrawal of appropriate amounts of NaCl (Heilbrunn, 1952). Compensation was made for added KCl by removal of equimolar amounts of NaCl.

All solutions were bubbled with 95% O_2 and 5% CO_2 in a reservoir and then passed, through a drip counter and heating coils in the water-bath, to the muscle chamber. A fine-pointed glass sucker connected to a Venturi pump drew the fluid across the preparation into a collecting flask. At the usual rate of flow (250–500 ml./hr) the temperature in the muscle chamber was 37–38° C. A thermometer in a side compartment of the chamber measured the temperature in all experiments. When higher rates of flow were used to produce rapid changes of the bathing solution, the fluid in the reservoir was heated to about 40° C. Temperatures between 30 and 40° C could be maintained accurately to within 0·5° C by variation of the temperature of the water-bath and of the reservoir of solution.

The pH of the solution, normally 7·3–7·4 at 37° C, was altered in some experiments by variation in the amount of added $NaHCO_3$. In three experiments an alkaline pH was required outside the range of a bicarbonate—CO_2 buffer. Tris (hydroxymethyl) aminomethane was used to give a final pH of 8·6 at 37° C. To keep 10 mM-$CaCl_2$ from precipitating at this pH it was necessary to omit both the $NaHCO_3$ and NaH_2PO_4 from the solution. When pH alterations were made, the final pH of the solution, bubbled with 'Carbogen' at 37° C, was measured with a pH electrometer.

Recording. M.e.p.p.s were recorded with conventional intracellular recording techniques (Ling & Gerard, 1949; Nastuk & Hodgkin, 1950). The micro-electrodes were filled with 3 M-KCl and had resistances between 5 and 20 MΩ. The method of pulling and filling these electrodes has been previously described, as have also the micromanipulators (Eccles, Fatt, Landgren & Winsbury, 1954) and electronic recording technique (Brock, Coombs & Eccles, 1952). The indifferent electrode in all experiments was a silver–silver-chloride spiral connected to the solution bathing the preparation by an agar–saline column.

Experimental procedure. The effect of magnesium was measured by comparing the frequency of m.e.p.p.s in the same muscle fibre before, during and after treatment with the magnesium-containing solution. When measuring the effect of calcium the reverse sequence was employed, i.e. solution with added calcium, solution without calcium, solution with added calcium. It was found that the preparation survived for a longer time, and in better condition when kept for two-thirds of the experiment in a calcium-containing medium. If the solution to be washed out contained 10 mM or more of calcium, the sodium salt of ethylene diamine tetracetic acid (EDTA), a chelating agent, was added to the calcium-free solution (in a 1 mM concentration). By this means the removal of calcium was completed in under 15 min, which was about half the time required in the absence of EDTA.

In each preparation the experimental sequence was followed at 8–16 neuromuscular junctions. Often several trials were made at each junction.

M.E.P.P. frequencies were initially measured by photographing successive oscillograph traces and counting m.e.p.p.s from film, usually with some form of magnification. Later a more convenient method of measurement was devised. After amplification the potentials were fed into a dekatron scaler (Isotope Developments Limited) using suitable cut-off bias to exclude base-line noise. The model used had a resolution time better than 3 μsec, and counted random potentials at a frequency of 3000/sec with only a 1% error. Special care was taken to avoid artifacts and keep base-line noise levels low. Frequencies were normally counted over 100 sec periods, but in solutions in which the potassium concentration was 10 mM or more the m.e.p.p. frequency in the presence of calcium was so high that 40 sec or even 10 sec counting periods gave satisfactory results. It was possible by this method to assess accurately when changes in frequency were fully established. A further advantage was that results were immediately available. In some experiments a photographic record was also made of the m.e.p.p.s. The results were in good agreement with those obtained with the scaler.

RESULTS

During the present investigation it was noticed that m.e.p.p.s could be recorded from muscle fibres bathed in 'calcium-free' solutions for more than 5 hr. In further experiments with calcium-free solutions m.e.p.p. frequencies at individual junctions were stable for up to half an hour, even in the presence of a chelating agent (EDTA) in concentrations up to 5 mM. Even when the calcium-free solutions contained 15 mM-$MgCl_2$, m.e.p.p.s were still recorded at frequencies within the normal range. This continuing release of transmitter, unaffected by wide variations in calcium and magnesium concentration, will be termed the fixed fraction of the spontaneous release. Upon this fixed fraction could be superimposed a fraction whose size is dependent on the concentrations of calcium and magnesium.

Effect of calcium

The effect of calcium-containing solutions on m.e.p.p. frequency was an increase in frequency from the basal level in the absence of calcium. Over the range of calcium concentrations explored (0·20–10 mM), the increase in m.e.p.p. frequency was linearly proportional to the logarithm of the bathing calcium concentration (Fig. 1). The increase in frequency was fully developed within 4–8 min of changing solutions. There was considerable variation in the time which elapsed before any changes in frequency occurred, but thereafter the development was so rapid that it was complete within 2 min.

The effect of calcium on m.e.p.p. frequency was explored in the presence of 0, 1 and 2 mM-$MgCl_2$. It was found that in each case a linear relationship existed between the logarithm of the bathing calcium concentration and the increase in m.e.p.p. The effect of any given calcium concentration was, however, reduced by about 20 % for each 1 mM increase in magnesium concentration (Fig. 1).

Effect of magnesium

In Table 1 the results of nine experiments on one preparation are summarized. They show that a magnesium concentration of 3 mM depressed the frequency of the spontaneous discharge of potentials by about 40 %.

It will be noticed that the m.e.p.p. frequency was not always the same before and after the period of immersion in 3 mM-$MgCl_2$. In order to take account of this a linear change in frequency with time has been assumed, and the average of the final and initial frequencies was compared with the frequency in the test solution (Column 5). A range from 1·0 to 15 mM-$MgCl_2$ was explored in this way in the presence of 2 mM-$CaCl_2$. The results (lowest line of Fig. 2) show that the depression was fully developed at 3 mM.

When the preparations were in solutions containing magnesium at 10 mM or higher concentrations, neuromuscular transmission was blocked and end-plate potentials were recorded in response to stimulation of the

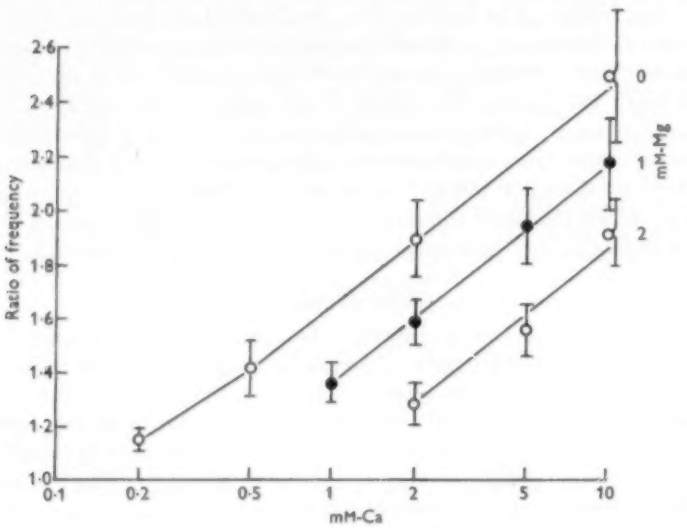

Fig. 1. Influence of calcium concentration upon m.e.p.p. frequency.

Ordinate. Ratio of frequency of m.e.p.p.s in calcium-containing solution to frequency in solution without calcium. Abscissa. Calcium concentration. Upper points obtained in absence of $MgCl_2$, middle in 1 mM-$MgCl_2$, lower in 2 mM-$MgCl_2$. Bars indicate ± 1 S.E. of mean ratio of frequencies.

TABLE 1. Effect of Mg on frequency of m.e.p.p.s

Measurements were made in solution with no added $MgCl_2$ (*A*) and in solution containing 3 mM-$MgCl_2$ (*B*). $CaCl_2$ 2 mM was present throughout. Frequency ratio is calculated from the arithmetic mean of the two *A* values.

Junction	Discharge rates (per second) *A*	*B*	*A*	Frequency ratio *B*/*A*
1	3·68	1·89	2·64	0·599
2	1·04	1·02	2·39	0·591
3	2·13	1·76	2·46	0·764
4	1·78	1·36	1·80	0·760
5	10·94	4·40	2·47	0·655
6	6·38	4·54	7·55	0·651
7	4·32	2·55	4·50	0·577
8	3·25	1·18	2·62	0·402
9	7·88	5·69	7·93	0·719
	Mean and S.E.			$0·64 \pm 0·04$
	Frequency as % of control			64 ± 4

phrenic nerve, even though the depression of m.e.p.p. frequency was then not greater than at lower concentrations of magnesium.

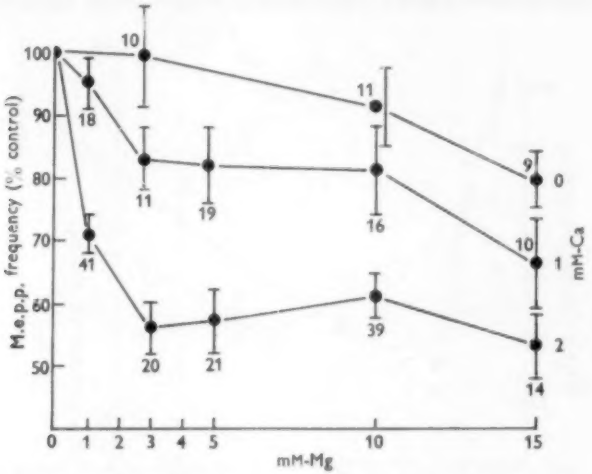

Fig. 2. Influence of magnesium concentration upon m.e.p.p. frequency.

Ordinate. M.e.p.p. frequency as a percentage of frequency in a solution without magnesium. Abscissa. Magnesium concentration. Upper points obtained in absence of $CaCl_2$, middle in 1 mM-$CaCl_2$, lower in 2 mM-$CaCl_2$. Bars indicate $\pm$ 1 S.E. of mean depression. Figures under points give number of junctions explored.

The reduction of frequency appeared within 2 min of changing solutions. The effect of temperature on the depression was investigated by repeating the experiments at 31·5–32° C. Over a range of magnesium concentrations from 1 to 10 mM the mean depressions of frequency found were within one standard error of the mean depressions found at 37–37·5° C. Similarly, when the effect of 10 mM-$MgCl_2$ was examined at pH 6·8 (bicarbonate buffer) and at pH 8·6 (Tris buffer), the mean results were almost identical with those at pH 7·4.

Interaction of calcium and magnesium

The results of the present investigation suggest that the action of magnesium was exerted solely by a reduction of the accelerating effect of calcium on m.e.p.p. frequency, as shown in Fig. 1. This would imply that magnesium should be without effect on m.e.p.p. frequency in the absence of calcium. Figure 2 shows that when the calcium concentration of the control and test solutions was reduced to 1 mM the effect of magnesium was greatly reduced over the whole concentration range (1–15 mM). More significantly, repetition of these experiments in the calcium-free

solutions showed that magnesium was indeed now without effect in all but the highest concentrations (uppermost curve of Fig. 2). The magnesium depression of frequency should thus be counteracted either by an absence of

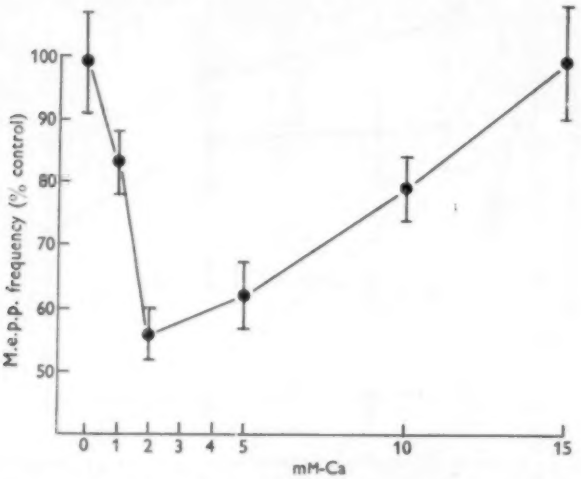

Fig. 3. Influence of calcium concentration on the depression of m.e.p.p. frequency by 3 mM-Mg.

Ordinate. M.e.p.p. frequency as percentage of frequency in a solution without magnesium. Abscissa. Calcium concentration. Bars indicate ± 1 S.E. of mean depression.

calcium or by sufficient excess of calcium to overcome the action of magnesium. This point was tested by exploring the depression of m.e.p.p. frequency by 3 mM-$MgCl_2$ in the presence of 0–15 mM-$CaCl_2$ (Fig. 3). As would be expected from the preceding results, the magnesium depression of frequency was, in fact, at a maximum close to 2 mM calcium.

Effect of depolarization of nerve terminals

The interaction of calcium and magnesium described in the preceding section is of the same type as that suggested by del Castillo & Katz (1956) for the action of these ions on transmitter release by depolarization of nerve terminals. This would suggest that the fraction of the spontaneous transmitter release that is affected by calcium and magnesium might be increased by depolarizing the endings. Raising the potassium concentration of the external medium and thus producing a stable depolarization of nerve terminals (Liley, 1956*c*) was a convenient way of testing this suggestion. The interaction of potassium and magnesium was explored by examining the effect of 3 mM-$MgCl_2$ on the m.e.p.p. frequency in the

presence of 1–20 mM-KCl (Fig. 4). Potassium would be expected to depolarize the nerve terminals appreciably only at concentrations of 10 mM or above (Liley, 1956*c*) and correspondingly there was statistically no

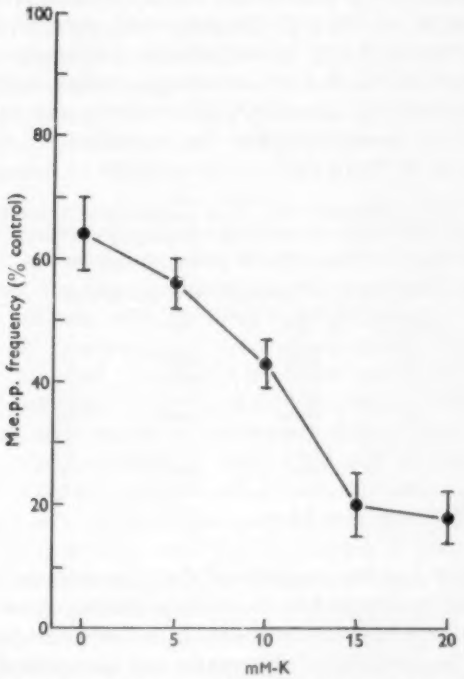

Fig. 4. Influence of potassium concentration on the depression of m.e.p.p. frequency by 3 mM-Mg.

Ordinate. M.e.p.p. frequency as percentage of frequency in solution without magnesium. Abscissa. Potassium concentration. Bars indicate ± 1 S.E. of mean depression.

significant difference between the depressions of frequency in 0 and 5 mM-KCl. The depressions of m.e.p.p. frequency produced by magnesium in the presence of 10, 15 and 20 mM-KCl were progressively much larger than the depression in the presence of 5 mM-KCl, suggesting that the action of magnesium was indeed dependent on the level of depolarization of the nerve terminals.

The accelerating action of calcium on m.e.p.p. frequency on this hypothesis should also be demonstrable in the presence of increased concentrations of potassium. To test this possibility the interaction of calcium and potassium was then explored in a slightly different way. Throughout

a series of experiments the potassium concentration was kept constant at 20 mM, and the effects of 2, 5 and 10 mM-$CaCl_2$ were compared with the effects of a solution containing no calcium but 1 mM EDTA. In six experiments of this type there was no doubt that in the absence of calcium the effect of potassium on m.e.p.p. frequency was greatly reduced. When calcium was introduced the acceleration of frequency was rapid and usually completed within 2–4 min of changing solutions (Table 2). But the mean frequency in 2 mM-$CaCl_2$ (1107 ± 102) was not significantly different from the mean frequency in 5 mM-$CaCl_2$ (1058 ± 46 for 10 measurements) or in 10 mM-$CaCl_2$ (1123 ± 115 for 11 measurements).

TABLE 2. Effect of Ca on frequency of m.e.p.p.s in presence of 20 mM-KCl. Measurements were made in solution with no added $CaCl_2$ but containing 1 mM EDTA (*A*) and in solution containing 2 mM-$CaCl_2$ (*B*). $MgCl_2$ 1 mM was present throughout

	Discharge rates (per second)		
Junction	*A*	*B*	*A*
1	3·60	1426·7	2·54
2	9·39	457·9	—
3	5·70	1206·6	7·5
4	9·09	1308·8	5·7
5	3·99	1205·75	4·61
6	3·31	1275·4	4·1
7	8·11	1069·8	3·45
8	7·12	904·5	2·66
Mean and S.E. in 2 mM-$CaCl_2$ (*B*)		1107 ± 102	—

The possibility was then considered that the increase in m.e.p.p. frequency produced by 20 mM-KCl was so large that an effect of calcium concentration on frequency would be masked. The experiments were therefore repeated with 10 mM-KCl, by both paired and randomized methods, with the same negative result. It is clear that variations in the calcium concentration over the range effective in the presence of 5 mM-KCl (Fig. 1) do not accelerate the m.e.p.p. frequency when the potassium concentration is raised to 10 or 20-mM. As the presence of some calcium is essential for the augmentation of spontaneous activity by potassium (Table 2) a dependence of m.e.p.p. frequency on calcium concentration might, under these circumstances, perhaps be found in a lower range.

Effect of osmotic pressure

Furshpan (1956) has shown that the m.e.p.p. frequency at the frog neuromuscular junction is very sensitive to changes in osmotic pressure and that this sensitivity is not affected by the presence of magnesium. In the present investigation an osmotic pressure difference between control and test solutions of up to 5 % affected neither the m.e.p.p. frequency nor the depression of the m.e.p.p. frequency by magnesium. Some experi-

ments were done with solutions made hyperosmotic by adding 10 mM-$MgCl_2$ without any withdrawal of NaCl in compensation ($CaCl_2$ was 2 mM in all solutions). When these solutions were compared with solutions without $MgCl_2$, there was a balance of two effects, the mean result at 37° C (10 mM–0 mM $MgCl_2$) being 0·98 (S.E. 0·11) for 12 measurements, i.e. there was neither an acceleration due to a 9% hyperosmotic solution nor a depression by the magnesium. Five experiments were done with magnesium in 9% hypo-osmotic solution. Here the effects of the two agents were additive, again indicating their independence.

DISCUSSION

The acceleration of m.e.p.p. frequency by calcium (Fig. 1) is of the same order as that described by Boyd & Martin (1956*a*) and Liley (1956*d*), and likewise there was great variability in the magnitude of the acceleration at different junctions with higher calcium concentrations. Magnesium and calcium both penetrate cell membranes slowly (Engbaek, 1952; Hodgkin & Keynes, 1957; Gilbert, 1960). The rapid actions on m.e.p.p. frequency described here are thus probably exerted at surface sites in the nerve membrane. The reciprocal relationships that have been demonstrated for the concentrations of magnesium and calcium on m.e.p.p. frequency (Figs. 1, 2, 3) suggest that both ions act at the same site.

As this action is affected by a potassium-induced depolarization of nerve terminals (Fig. 4) it is likely that the mechanism of transmitter release studied in the present investigation is the same as the voltage-dependent, calcium–magnesium sensitive mechanism proposed by del Castillo & Katz (1956) and Liley (1956*c*) to explain release of transmitter by nerve impulses. Thus, in this preparation the membrane potential must be at a level allowing limited activity of this mechanism. Hyperpolarization of the nerve terminals would then be expected to reduce this activity and thereby to reduce m.e.p.p. frequency, as Liley (1956*c*) has indeed found.

It appears likely that the depressive effect of magnesium on m.e.p.p. frequency described here, was not found by Boyd & Martin (1956*a*) in the cat tenuissimus muscle, because they compared the effect of a solution containing 1·15 mM-$MgCl_2$ with one containing 9·37 mM, both being in the presence of 2·46 mM-$CaCl_2$. The results of the present investigation (Fig. 2) indicate that there would be only a 10% increase in depression in the higher magnesium concentration. Such a small difference would be difficult to detect if only a small number of junctions were examined. In fact, when solutions containing 1 mM-Mg and 10 mM-$MgCl_2$ were compared (in the presence of 2 mM-$CaCl_2$) in the present investigation, no frequency change was found. (Mean ratio of frequencies at sixteen junctions 10 mM–1 mM-$MgCl_2$ was 1·00, S.E. $\pm$ 0·10.)

The absence of any effect of magnesium on m.e.p.p. frequency in the frog (del Castillo & Katz, 1954*a*) cannot be explained in the same way. In these experiments the effect of solutions containing no magnesium and 16·2 mM-$MgCl_2$ were compared. In the present investigation such a change in concentration would have reduced m.e.p.p. frequency at least 45% (Fig. 2). This difference may indicate that in amphibia the level of the membrane potential of nerve endings is such that activity of the voltage and calcium–magnesium sensitive system is not demonstrable. If this were so, the negligible reduction of m.e.p.p. frequency obtained when nerve terminals are hyperpolarized in frog nerve-muscle preparations would also be explained (del Castillo & Katz, 1954*b*; Kraatz & Trautwein, 1957). A further question raised by this species difference is whether the difference is physiological or merely a reflexion of a greater difficulty of maintaining rat diaphragm preparations in good condition. Similar effects on m.e.p.p. frequency, with calcium at least, have been obtained in the cat tenuissimus preparation (Boyd & Martin, 1956*a*); therefore this criticism cannot be confined to the rat diaphragm. In this connexion it might be expected that, during the course of experiments, as the preparation aged the effect of magnesium would be progressively increased. There was, however, no consistent trend of this kind when results from the first and last two junctions in many experiments were compared. Thus, if there was a depolarization of nerve terminals it did not increase appreciably during the experiments.

The interaction of osmotic pressure and magnesium in the present investigation indicated that these agents affected m.e.p.p. frequency by different mechanisms. In the terms used previously, osmotic pressure increases the fixed fraction, while magnesium affects the voltage and calcium-sensitive fraction. Post-activation potentiation and stretching of the preparation, which increase m.e.p.p. frequency despite concomitant high magnesium concentrations (Liley, 1956*b*; Hutter & Trautwein, 1956), must also act independently of the release mechanism studied in this investigation.

SUMMARY

1. Intracellular recording from neuromuscular junctions in isolated preparations of rat diaphragm has revealed that miniature end-plate-potential frequency is accelerated by calcium and depressed by magnesium. This action appears to be exerted at surface sites in the presynaptic membrane. The action of magnesium is explained as a competition with calcium.

2. Miniature end-plate potentials can still be recorded in the absence of calcium or the presence of high concentrations of magnesium.

3. The mechanism of the fraction of spontaneous transmitter release

which is affected by calcium and magnesium is thought to be the same as that activated by nerve impulses.

The author wishes to acknowledge the constant encouragement and helpful criticism of Professor J. C. Eccles during the course of this investigation.

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THE HEAT PRODUCED BY A MUSCLE AFTER THE LAST SHOCK OF A TETANUS

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In his last published paper (1933) on the heat production of muscle, before he turned over to calculations in mathematical physics (see Hill 1943), W. Hartree gave two figures (his Figs. 3 and 4) which showed the rate of heat production during and after contractions at 0 and 17° C respectively. These are reproduced here as Fig. 1 with minor changes, as an introduction to the present paper. At 0° C the stimulus was of 0·6 sec, the heat after the last shock was 0·430 of the whole initial heat, and it had ended by the time the tension had fallen to zero. At 17° C the stimulus was of 0·3 sec, the heat after the last shock was 0·354 of the whole, and it continued for a long time after the muscle had relaxed. Hartree did not report the absolute amounts of heat, but in frog sartorii in good condition the total initial heat could not have been far from: in 0·6 sec tetanus at 0° C, 7·5 mcal/g muscle; in 0·3 sec tetanus at 17° C, 15 mcal/g muscle. The heat therefore after the last shock must have been, at 0° C about 3·2 mcal/g, at 17° C about 5·3 mcal/g.

Hartree's instruments by 1933 had been considerably improved and should have been quick enough to give a reasonably accurate analysis of the heat production at 17° C; for 0° C they were certainly adequate. Two questions arise from a comparison of his results at the two temperatures: (1) why did the heat production continue so long at 17° C after the tension had fallen to zero, whereas at 0° C it had ended when relaxation was complete? (2) why was the heat produced after the last shock so much greater at 17° C than at 0° C?

The apparent result at 17° C, that the heat continued after the muscles had relaxed, had been found before by Hartree & Hill (1928, see Fig. 2) and particularly by Hartree (1932), though the methods then used were not so good as in 1933. The continuing heat production after the muscle had relaxed had nothing to do with the ordinary recovery heat, which starts gradually and a second or two later, except after long stimuli when it can merge into the initial heat. Could it be a sign of the Lohmann reaction, $ADP + CrP \rightarrow ATP + Cr$, in which ATP is re-formed with the aid of free energy provided by the splitting of phosphocreatine? Un-

fortunately the heat produced in this reaction in living frog muscle at normal pH is not accurately known, but it is probably positive. If the reaction occurred during contraction, or in the interval between the last shock of a tetanus and the disappearance of tension, there would be no way of separating its heat from that of other processes going on at the

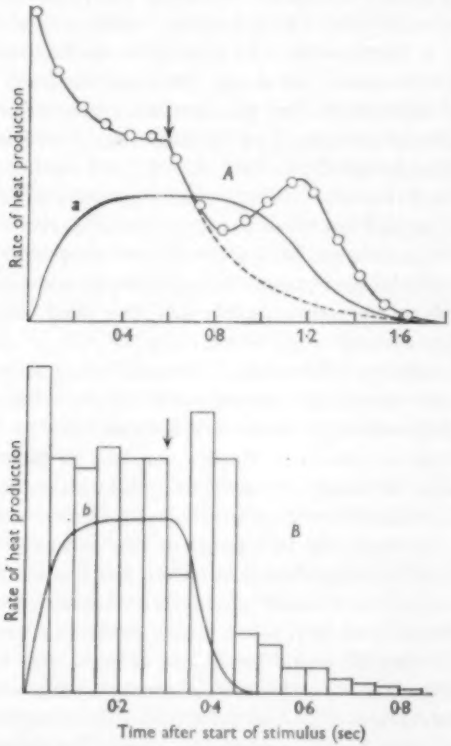

Fig. 1. Rate of heat production during and after isometric contractions of frog sartorii. *A*, 0·6 sec stimulus at 0° C, heat analysed in 0·1 sec units; *a* tension. *B*, 0·3 sec stimulus at 17° C, heat analysed in 0·05 sec units; *b* tension. Abscissae, time from start of stimulus; ends of stimuli shown by arrows. The interrupted line in *A* gives the estimated rate of the heat of activation after the last shock. Modified from Hartree (1933, Figs. 3 and 4).

same time. If, however, it continued after the muscle had relaxed but before the normal recovery heat began, its heat should be distinguishable. Any evidence which could help to clear up the current acute uncertainty about the chemistry of contraction in living muscle would be valuable, and the present investigation was started with that purpose in mind.

Attention was concentrated experimentally on the heat produced after the last shock of a tetanus, at 0° C and at room temperature: but at room temperature the records were usually continued until the recovery heat began to appear. At 0° C the heat production never lasted beyond the end of relaxation; and the heat produced after the last shock was usually not more than about 3·5 mcal/g. At room temperature, however, the results were very variable. In toad muscles, which in every respect except speed are similar to frog muscles, the heat after the last shock was seldom much prolonged and never very large. In a special series of five experiments with toad sartorii at room temperature three showed no heat production at all after the tension had fallen to zero, and the heat after the last shock averaged 3·6 mcal/g: in one only did any significant heat appear after relaxation was complete. In several experiments with frog sartorii the heat production did not continue long after the tension had fallen to zero, and the total amount of heat after the last shock was not very large. In others, however, the heat production apparently continued for a second or more after relaxation was complete and the total amount of it after the last shock was excessive, up to 9 mcal/g.

With all this variation between individual muscles, and the fact that in slower muscles (toads', or any muscles at 0° C) the effect was smaller or absent, it was impossible to draw any firm conclusion about chemical processes following contraction. Rather one had to examine the experiments and material critically, to see if any technical cause could explain the variation. The older thermopiles (1932 and earlier) were not nearly so flat as those in use now; and in a paper on the initial heat production of muscle Hartree (1931) suggested that 'when the muscle tightens (during isometric contraction) the thermal conductivity between it and the thermopile may be different from that when taking controls. Any depressions on the face of the thermopile would tend to be bridged over by the muscle as the tension rises'. If a layer of fluid of average thickness (say) 0·1 mm were to lie between the muscle and the thermopile, it would greatly hinder thermal conduction from the one to the other. The consequence of this would be that heat which was really produced during contraction would appear to be produced later. With the very flat thermopiles now employed this could hardly be expected to happen, unless exceptionally and owing to some special cause. But the same effect could result from an uneven distribution of the heat produced during contraction. If the rise of temperature in a region of the muscle distant from the thermopile were greater than the average, the final rise of temperature *at* the thermopile would be correspondingly delayed.

The possibility, therefore, of the actual occurrence of a non-uniform production of heat in a contracting muscle had to be tested experimentally.

This could be done by comparing pairs of records of the heat production made with a single muscle mounted (*a*) in the usual way on a thermopile, i.e. with the epimysium side inwards and (*b*) the other way round, with the body side inwards. If a difference was found between the two records of a pair this could only be due to the muscle producing heat unequally in its opposite halves: for both sides should make an equally good thermal contact with a thermopile. In some experiments the two records were nearly alike, but in others they differed substantially.

It was clear, therefore, that heat can in fact be produced non-uniformly during contraction; so a mathematical and numerical investigation was undertaken to find out whether such irregularities could explain the anomalies observed. It was possible to show, on easily admissible assumptions of the distribution of the heat produced during contraction, that they could. The calculations proved, moreover, that in the case of the much slower heat production of muscles stimulated at 0° C similar anomalies would not be evident even if massive irregularities of distribution occurred: they showed also that in the slower muscles of toads the anomalies would be smaller and less obvious than in the faster muscles of frogs. This curious result is really determined by the ratio of the rate of heat production during contraction to the rate of heat conduction. The former varies with temperature or with species of animal, the latter is practically constant.

All this led to a closer consideration of the nature and magnitude of the 'relaxation heat' after an isometric contraction. For many years the 'hump' in the relaxation heat (see Fig. 1 A, above) was attributed to the degradation into heat of the elastic energy developed during contraction. Recently, however, it became apparent that the elastic energy was not large enough to account for the whole of the heat in the 'relaxation hump'; and at about the same time it was realized that the release of tension by itself causes a substantial production of heat (the 'thermoelastic' heat). The elastic energy and the thermoelastic heat together could provide about 2 mcal/g, which was enough to explain the 'relaxation hump'. But the heat produced after the last shock of a tetanus, when the distribution of heat had been uniform, was about twice this amount, so a further 2 mcal/g had to be looked for. This led to a consideration of the amount of 'activation' (or 'maintenance') heat still remaining to be produced following the last shock: and two independent methods of estimating it led to the conclusion that it could provide about the amount required.

METHODS

The muscles used were a pair of sartorii, either of frog (*Rana temporaria*) or of toad (*Bufo bufo*). They were mounted on thermopile D_1, shown in Fig. 1 of a paper by Abbott, Aubert & Hill (1951). In nearly all respects experimental details were the same as those

described in a recent paper (Hill, 1961). Care was taken that contractions should be as isometric as possible; to this end a strong cross-bar was fixed between the side arms of the thermopile frame, and to this the threads for the tibial tendons could be firmly tied. In eight special experiments records of tension were required as well as of heat. Then thermopile D_3 was used, and the muscles were joined by a wire to a transducer valve (RCA 5734). Each thermopile had a half thickness equivalent to about 17 μ of muscle; together with the epimysium and adhering fluid this gave an estimated value of 22·4 μ for the constant b (see Hill, 1949*c*). This was used in computing the factors required for the analysis of the heat production (Hill, 1949*a*, *c*). The average thickness of each pair of muscles used was between 550 and 750 μ.

It was important in these experiments to maintain the muscles in as constant a condition as possible, and for most of the time they were kept immersed in Ringer's solution in the thermopile chamber. In order to run the solution in and out with a minimum of thermal disturbance, the arrangement described by Hill & Howarth (1957, p. 22) was employed. The usual procedure was to withdraw the solution about 5 min before a record of the heat production was to be made: it was replaced automatically by moist oxygen at the same temperature. Then after the muscle had been stimulated and the heat recorded the solution was run in again and left with oxygen bubbling through until about 5 min before the next record.

It was essential to the purpose of the experiments that a clear signal should appear on the heat records to mark the moment when the last shock of a tetanic stimulus was applied. This was provided by a slight dimming of the beam of the cathode-ray tube from the moment when the stimulating current was short-circuited. Stimulation was effected by a series of rapid condenser discharges provided by a Carpenter relay. This was operated by an Attree stimulator (Attree, 1950) locked to a frequency corresponding to 1, 2, 3, 4 or 5 cycles of the power mains (50 c/s). This stimulator also provided the time spots on the trace.

The amount of heat produced was generally so large that considerable negative feedback could be applied to the galvanometer. This made it very rapid, and usually there was no need of any allowance for delay in its deflexion. The allowance for heat loss was applied by using the exponential time constant obtained from a record of artificial (electrical) heating. The heating was by an alternating current of 50 kc/s, a frequency high enough not to stimulate, so the 'heating controls' could be made at any appropriate time on the living muscle.

The analysis, providing the allowance for time lag in the conduction of heat into the thermopile, was continued until the analysed result became constant, before the ordinary recovery heat production began.

In a few experiments heat records were made with the muscles in nitrogen as well as in oxygen, but no significant difference was found.

RESULTS

The heat produced after the last shock of a tetanus

The results at 0° C can be simply stated: they were in fact very like Hartree's, shown in Fig. 1*A* above. In frog sartorii the heat production ended in about 1·2 sec (after the last shock), i.e. the time required for the tension to fall to zero: it was about 3·0 mcal/g. In toad sartorii the heat production ended within 2·0 sec, by which time relaxation was complete: in four experiments, with 3 or 4 sec tetani, the amount after the last shock averaged 2·9 mcal/g. These quantities, as will be shown below, can

be fully accounted for in terms of elastic energy, thermoelastic heat and the residue of maintenance, or activation, heat.

At room temperature the results with toad muscles were as follows. In five consecutive experiments with rather small muscles at 16·6° C, with tension recorded as well as heat, three gave no heat at all after the tension had fallen to zero; one gave 0·1 mcal/g, the fifth 0·9 mcal/g, with a total after the last shock of 5·1 mcal/g. The last experiment is considered below in connexion with Fig. 7, where it is shown that the whole of the excess heat can be attributed to a small uneven distribution during contraction. In an earlier experiment with larger toad muscles at 20° C the following results were obtained. These muscles were remarkably good, constant results being obtained over many hours. With stimuli ending at 0·2, 0·4, 0·76, 1·6 and 3·2 sec, the heat produced after the last shock, up to the moment (0·29 sec later) at which, at 20° C, the tension should have fallen to zero, was 4·2, 4·4, 4·3, 3·8 and 4·1 mcal/g, respectively, mean 4·15 mcal/g. After that, in the next 1 sec or so, a small amount of extra heat appeared, 0·8, 0·9, 1·2, 0·9 and 0·9 mcal/g. It will be shown below (p. 533) that the whole of the extra delayed heat can be attributed to a small uneven distribution.

The three experiments with toad muscles referred to above, in which the heat production had ceased by the time the tension had fallen to zero, agreed well with one another; and the muscles were certainly very good, because the values of Pl_0/M were high, 2690, 2800, 2770, average 2750 (P = tension developed (g wt.); M = mass of muscle (g); l_0 = standard length (cm)). The heat was analysed in 40 msec time units, and the mean result is given in Fig. 2, the rate of heat production being shown by horizontal bars from 0·12 sec before the last shock (at 0·48 sec) to 0·48 sec after it. The mean tension also is given, dropping to zero at about 0·32 sec after the last shock. The heat after the last shock was 3·6 mcal/g. The results represented in Fig. 2 are considered again below (p. 535) in the section dealing with the relaxation heat.

With frog sartorii at room temperature the results were far more variable. Rather large muscles were used, in which (as will be shown below) the effect of an uneven distribution of the heat produced during contraction would be greater. Figure 3 gives in graphical form the analysed results of 13 contractions in 6 experiments at about 17° C, with durations of stimulus from 0·2 to 2·0 sec. The solid portion of each vertical bar gives the amount of heat produced between the last shock and a moment 0·2 sec later when the tension should have fallen to zero. It is very inconstant, varying from 3·9 to 6·8 mcal/g; its inconstancy is the more evident when we remember that it contains a large nearly constant component made up of the sum of elastic energy, thermoelastic heat and the residue of the

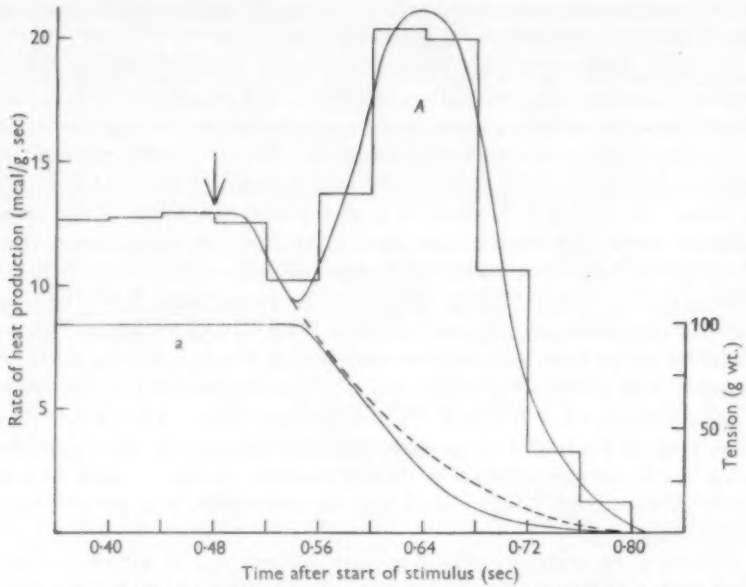

Fig. 2. Rate of heat production (A) and tension (a) during and after an isometric contraction of toad sartorii at 16·6° C. Stimulus 0·48 sec at 25 shocks/sec, last shock shown by arrow. Heat analysed in 0·04 sec units. The interrupted line below A gives the estimated rate of the heat of activation after the last shock.

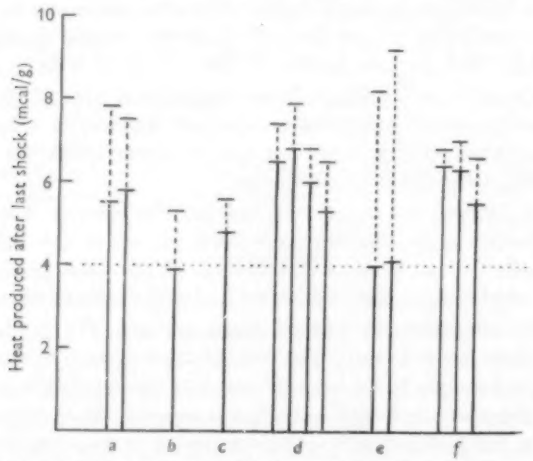

Fig. 3. Heat produced by frog sartorii in isometric tetanic contractions of various durations at room temperature (about 17° C). Six experiments, a–f, with one to four contractions in individual experiments. Solid lines, heat produced between last shock and end of relaxation: interrupted lines, heat produced after end of relaxation. The horizontal dotted line is drawn at height 4 mcal/g.

maintenance heat. The dotted horizontal line at height 4 mcal/g represents the expected value of this constant component at 17° C, and the variation above this level is from $-0{\cdot}1$ to $+2{\cdot}8$ mcal/g.

The upper, interrupted, portion of each vertical bar represents the heat produced after relaxation was complete, i.e. after 0·2 sec from the last shock. It was very inconstant, varying from 0·4 to 5·1 mcal/g.

The results of experiment (*e*), Fig. 3, which gave much the largest amount of heat after the end of relaxation, are examined in detail below in connexion with Fig. 8. Three typical results from among the rest, in

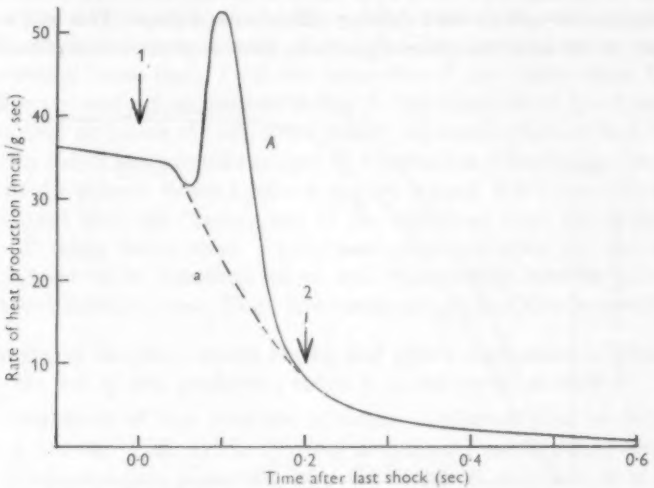

Fig. 4. Rate of heat production (*A*) after the last shock (arrow 1 at time '0') of an isometric tetanus (0·32 sec) of frog sartorii at 17° C. Mean of three experiments. Arrow 2 shows the usual time (0·2 sec after the last shock) when, at 17° C, the tension falls to zero. Line (*a*) gives the estimated rate of activation heat after the last shock.

which the heat was analysed in 0·04 sec time units, were averaged and are presented in Fig. 4. The rate of heat production is given from 0·10 sec before the last shock to 0·60 sec after it. The position of the last shock and the presumed moment at which the tension had fallen to zero are indicated by arrows. The total heat after the last shock was 7·4 mcal/g; the heat produced between the two arrows was 6·25 mcal/g.

In four of the six experiments of Fig. 3, namely (*a*), (*d*), (*e*) and (*f*), the length of the tetanus was varied, the later vertical bars of each group corresponding to the longer stimuli. The latter gave little or no more delayed heat than shorter stimuli, confirming a similar conclusion from

experiments with toad muscles. It is shown below that the apparent delayed heat due to an irregular spatial distribution of the rate of heat production during contraction would in fact be nearly independent of the duration of the stimulus.

Direct evidence for a non-uniform distribution of the heat produced during contraction

An easy way of obtaining a non-uniform distribution of the heat produced in a contracting muscle was to use a submaximal stimulus. A comparison of photographic records of the heat production with maximal and submaximal stimuli showed striking differences of shape This had nothing directly to do with the present problem, since supermaximal stimuli were

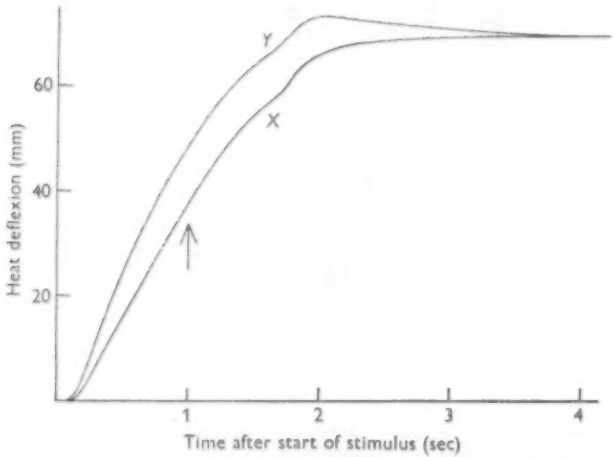

Fig. 5. Galvanometer deflexion due to heat production in a single frog sartorius at 0° C. 1 sec stimulus (end shown by arrow). Curve *X* was obtained when the 'outside' of the muscle was in contact with the thermopile, curve *Y* when the muscle had been reversed.

always used; but the comparison gave a vivid idea of the effect, on heat records, of non-uniform contraction. A direct way of exhibiting the irregularities that can occur when the stimulus is maximal is illustrated in Fig. 5. There *X* and *Y* are records, corrected only for heat loss, of the heat production of the same muscle, a single frog sartorius, on the same thermopile and under identical conditions—except that, for *X* the 'outside' of the muscle was in contact with the thermopile, for *Y* the 'inside'. By 'outside' is meant the side which, in the living animal, is next to the skin. The conditions were: 0° C; contraction rigidly isometric at length l_0;

stimulus direct, 11 supermaximal condenser discharges, the last at 1·0 sec. The only plausible explanation of the difference between records X and Y is that the 'outside' of the contracting muscle produced less heat than the 'inside'.

The difference between records X and Y does not look very large, but allowance must be made for the fact that equalization of temperature was going on all the time during contraction. The analysis described below was applied to the difference between the two records, and then it was found that a large part of all the heat produced must have occurred in a non-uniform distribution.

Six other experiments of the same kind were made with single frog sartorii. In one of them, records X and Y were almost identical; in one, X rose rather faster than Y; in the other four Y rose faster than X, but the difference was not as great as in Fig. 5. The usual effect, confirmed by the fact that an excess of heat, not a deficit, appeared after the last shock, is that the rise of temperature caused by contraction is less in the 'outside' region of the muscle. When a pair of sartorii is used, this is the side which is in contact with the thermopile. If the difference were the other way round (X rising faster than Y) the heat produced after the last shock would appear to be less than usual, and none would turn up after the tension had fallen to zero. There was never any sign of this happening.

The flow of heat in a muscle during and after a contraction in which the rate of heat production varies from one region to another

The treatment of this problem is rather similar to that of diffusion within a muscle (Hill, 1928). Several simplifying assumptions must be made. Each of a pair of sartorii on a thermopile is regarded as a plane sheet of uniform thickness a cm, insulated against flow of heat at its two faces. At its inner face, in contact with the thermopile, no heat can flow through the thermopile, because a similar muscle lies symmetrically on the other side: while allowance is made in the analysis for the (small) heat capacity of the thermopile and for lateral heat flow along its wires. Into the gas at the outer face the flow of heat is negligible in the times considered (1–3 sec). The problem thus is reduced to linear conduction of heat perpendicular to the plane of the sheet, with zero loss at both faces. Under such conditions the course of temperature change is governed by the equation

$$\partial y/\partial t = k\partial^2 y/\partial x^2. \qquad (1)$$

Here y is temperature, x is distance (cm) normal to the boundary surfaces, t is time (sec) and $k = K\rho c$ (K is thermal conductivity of muscle, ρ is its density and c its specific heat). According to Hill (1949*c*, p. 231) the value of k for frog muscle at 0° C is about $1{\cdot}25 \times 10^{-3}$. It depends chiefly on the

thermal conductivity of water, which increases only 6% between 0 and 20° C, so it will be taken as independent of temperature. It is convenient to express y in units of mcal/g muscle, instead of °C × 10^{-3}. This does not affect equation (1), for both sides can be divided by c and y/c is temperature in °C × 10^{-3}. For economy of words, therefore, y will be referred to as temperature.

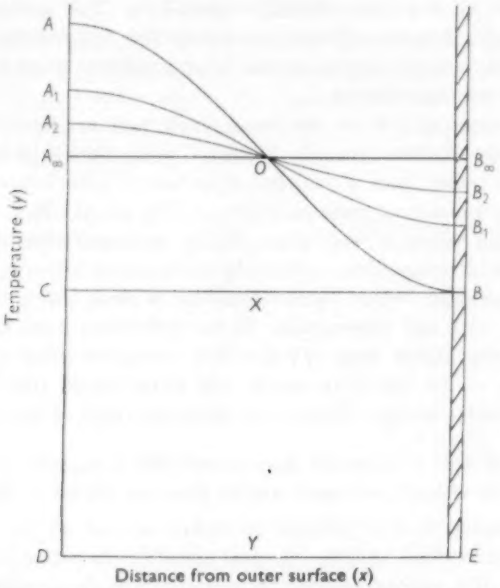

Fig. 6. Diagram to illustrate an initial non-uniform distribution of temperature in a muscle lying on a thermopile, and the manner in which it equalizes itself with time. Thermopile to the right, shaded. Total thickness of muscle and thermopile 0·641 mm. Initial distribution AOB, above the line DE; distribution after 0·231 sec, A_1OB_1; distribution after 0·462 sec, A_2OB_2; final distribution, $A_\infty OB_\infty$. The initial distribution AOB above the line CB is described as the non-uniform distribution, the distribution CB above the line DE as the uniform distribution.

Let x be measured from the outer surface of the muscle, so that the thermopile is at $x = a$. It is assumed that the temperature is a function of x and t only, so that an initial instantaneous distribution of temperature is a function only of x. It is not necessary to consider any uniform distribution of temperature; the non-uniform distribution is regarded as superimposed on the other (see Fig. 6). Take the rate of the non-uniform heat production as $uf(x/a)$, where u is a rate of heat production (in units of mcal/g.sec), and $f(x/a)$ is a number depending on the relative distance x/a from the outer surface. For simplicity in most of the calculations, since

the rate of heat production during a maintained contraction decreases only slowly, u will be taken as constant; and $f(x/a)$ is taken as zero at $x/a = 1$, i.e. at the surface of the thermopile.

For an instantaneous distribution of temperature $y = hf(x/a)$ at $t = 0$ the complete solution of equation (1) at all subsequent times is given by Carslaw & Jaeger (1959, p. 101, eqn. (6)). This, in general, involves an infinite series, though it is usually rapidly convergent. In one special case, however, which is convenient for the present discussion, the solution is simple: when $f(x/a) = (1+\cos \pi x/a)$ the solution is

$$y/h = 1+(\cos \pi x/a)e^{-k\pi^2 t/a^2}. \tag{2}$$

In Fig. 6 the initial non-uniform distribution at time $t = 0$ is the half sine-curve AOB above the line CB. The uniform distribution is CB above the line DE. If the muscle be 0·0641 cm thick (CB or DE), so that $k\pi^2/a^2 = 3$, the distribution at subsequent times is A_1OB_1 at $t = 0{\cdot}231$ sec, A_2OB_2 at $t = 0{\cdot}462$ sec, and $A_\infty OB_\infty$ at $t = \infty$.

Since we are concerned experimentally only with the temperature at the inner surface, where the thermopile is, i.e. at $x/a = 1$, the equation is further simplified (putting $k\pi^2/a^2 = \alpha$) to

$$y/h = 1-e^{-\alpha t}. \tag{3}$$

This represents the temperature at the thermopile at time t after an initial instantaneous distribution of temperature $y/h = 1+\cos \pi x/a$ at $t = 0$. In practice, however, we are not concerned with the effects of an initial instantaneous distribution of temperature, but with those of a continuing production of heat. The latter can be deduced from the former as follows. We assume a rate of heat production $u(1+\cos \pi x/a)$, so if u is constant the temperature y of the thermopile at time t after the start is

$$y = u\int_{\theta=0}^{\theta=t}(1-e^{-\alpha(t-\theta)})d\theta$$

$$= u\{t-(1/\alpha)(1-e^{-\alpha t})\}. \tag{4}$$

The quantity

$$(u/\alpha)(1-e^{-\alpha t}) \tag{5}$$

can be called the deficit of temperature at the thermopile at any moment, that is, the amount by which the temperature at that moment is less than it would have been if the rate of heat production had been uniform throughout the muscle, i.e. u instead of $u(1+\cos \pi x/a)$.

So far we have assumed u to be constant up to time t: but if the heat production were to end, i.e. if u became zero, at time t_1 ($< t$) the temperature at a subsequent time t would be

$$y = u\{t_1-(1/\alpha)(e^{-\alpha(t-t_1)}-e^{-\alpha t})\}. \tag{6}$$

The deficit of temperature, therefore, at time $t = t_1$ sec, would be

$$(u/\alpha)(1-e^{-\alpha t_1}), \tag{7}$$

and at time $t = (t_1+t_2)$ sec

$$(u/\alpha)(e^{-\alpha t_2}-e^{-\alpha(t_1+t_2)}). \tag{8}$$

Equation (7) then gives the total apparent heat production (in mcal/g) after $t = t_1$ sec, and equation (8) the total apparent heat production after $t = (t_1+t_2)$ sec.

The experimental results reported earlier in this paper refer to the total heat actually or apparently produced (i) after the last shock and (ii) after the tension had fallen to zero. The former (i) includes the heat due to elastic energy and thermoelastic heat, together with the residue of the maintenance heat after the last shock: the rest is believed to be an error due to a non-uniform distribution of the heat produced earlier during contraction. The latter (ii) is believed to be entirely an error due to the same cause. For simplicity in comparing the experimental results with equations (7) and (8) let us regard the real heat production after the last shock as uniformly distributed, so it does not come into the equations, and concentrate on the after-effects of a non-uniform distribution of the heat rate before the last shock. Then equation (7) gives the amount by which the observed heat production after the last shock is erroneously in excess of the true value, while equation (8) gives the error remaining at the moment when relaxation is complete.

Table 1 has been calculated from equations (7) and (8) for a number of typical cases. A moderate value has been taken, namely 1/3, of the ratio of the heat in the non-uniform distribution to the whole heat: this is the ratio (OX/OY) illustrated in Fig. 6. Three different experimental objects have been chosen, frog muscle at 17° C, toad muscle at 17° C and frog muscle at 0° C, in order to illustrate how greatly the size of the error depends on the speed of the muscle. Three different thicknesses of muscle have been considered, in order to show how much greater is the error in a bigger muscle: and to make the calculation more realistic a longer stimulus has been assumed for the muscle at 0° C.

The comment might be made that the calculated quantities in Table 1 depend directly on the value of v, so naturally vary with the muscle considered. That is true: but they are intended to be compared with the real heat production after the last shock, which is not far from constant at about 3–4 mcal/g. The error, therefore, varies from about 100 to 0% of the true quantity.

Five conclusions can be drawn from Table 1:

(1) in a fast muscle at room temperature the error can be large;

(2) in a slower muscle at room temperature it is less;

(3) in a muscle at 0° C, which is very slow, the total error after the last shock is small, after the end of relaxation negligible;

(4) the error is much less in thinner muscles, in which temperature equalization is quicker;

(5) the error tends to a constant value as the duration of the stimulus is increased.

TABLE 1. Apparent heat production after the end of the real heat production, caused by a non-uniform spatial distribution of the heat produced during a contraction; calculated from the formulae (7) and (8) in the text, for the following conditions:

v = total rate of heat production during contraction (mcal/g. sec);

$u = v/3$, where u is the rate of heat production in the non-uniform distribution $u(1+\cos \pi x/a)$;

t_1 = duration of heat production (sec);

t_2 = time after t_1 for tension to fall to zero (sec).

Muscle	Frog 17° C	Toad 17° C	Frog 0° C
v	30	12	4
u	10	4	1·33
t_1	1·0	1·0	3·0
t_2	0·2	0·32	1·2

Muscle size	A, rather large	B, medium	C, small
Thickness a (cm)	0·0702	0·0593	0·0497
$\alpha = k\pi^2/a^2$	2·5	3·5	5·0

Calculated results

Muscle	Temp (°C)	Size	Apparent heat production (mcal/g) after $t = t_1$	after $t = t_1 + t_2$
(1) For conditions described above				
Frog	17	A	3·66	2·22
Toad	17	A	1·47	0·65
Frog	0	A	0·53	0·03
Frog	17	B	2·77	1·37
Toad	17	B	1·10	0·36
Frog	0	B	0·39	0·00
Frog	17	C	1·99	0·72
Toad	17	C	0·80	0·16
Frog	0	C	0·27	0·00
(2) For frog muscle, 17° C, size B, when t_2 = 0·2 sec, t_1 varies				
t_1 (sec)				
0·5			1·79	1·17
1·0			2·77	1·38
1·5			2·84	1·41
2·0			2·86	1·42

Of these conclusions, results similar to (1), (2), (3), and (5) have been noticed continually in the experiments reported above: (4) was not noted with certainty, but the variation in size of the muscles used was not very large, and variations from other causes could have masked its effect. As

regards (5), the calculated result in Table 1 for $t_1 = 0{\cdot}5$ sec, is substantially less than for greater values of t_1. This inequality was not evident in the experimental results. But in actual contractions the rate of heat production is greater at the start and then soon falls to a nearly constant level: in the calculation the rate of heat production, for simplicity, was assumed constant throughout. It is possible by numerical integration to calculate the error for any variation of u with time. This was done for the case of an actual experiment in which the rate of heat production was known throughout a contraction with 2 sec stimulus. The calculated errors in the heat produced after the last shock were, in arbitrary units; after 0·5 sec, 4·0; after 1·0 sec, 4·2; after 1·5 sec, 3·9; after 2·0 sec, 3·6. These are nearly enough constant, and illustrate the experimental finding.

The assumption therefore of a non-uniform spatial distribution of the rate of heat production during a contraction is capable in general of explaining the kind of variations, anomalies and regularities that turn up in measurements of the heat production after the last shock of a stimulus. It will naturally occur to a reader that the distribution chosen, namely $(1+\cos \pi x/a)$, is arbitrary, and that another distribution might give a different result. In fact it does not. It is possible, for example, provided one is prepared for enough arithmetic, to try other distributions, for example $f(x/a) = 2$ from $x/a = 0$ to 0·5, and $f(x/a) = 0$ from $x/a = 0{\cdot}5$ to 1·0: this would mean that in the non-uniform distribution heat was produced only in the outer half of the muscles. The solution in this case for the temperature at the thermopile, in place of equation (3) above, is

$$y/h = 1-(4/\pi)\{e^{-at}-\tfrac{1}{9}e^{-9at}+\tfrac{1}{25}e^{-25at}\ldots\}. \qquad (9)$$

The effect of using this equation instead of (3) above is that the same error results from a somewhat smaller non-uniform distribution of the heat production. It was applied, with good effect, in explaining the results of the most anomalous experiment made (see Fig. 8 below). Another possible distribution is a linear diminution of temperature from the outside inwards, $f(x/a) = 2(1-x/a)$. The solution for this case is

$$y/h = 1-8/\pi^2\{e^{-at}+\tfrac{1}{9}e^{-9at}+\tfrac{1}{25}e^{-25at}+\ldots\}. \qquad (10)$$

The effect of using equation (10) instead of (3) is the opposite of that of using equation (9), namely that a rather larger non-uniform distribution of the heat production is required to produce the same error.

In general, therefore, the proposition seems to be proved. It only remains to see how well it works in specific experiments. In Fig. 7, curve *A* represents the heat production of a pair of toad sartorii during and after an isometric tetanus of 0·44 sec duration at 16·6° C. Curve (*a*) is the later part of the tension record. The last shock is shown by the arrow. The

tension had fallen to zero at 0·72 sec but the heat production continued at a diminishing rate for more than 1 sec longer. This was one of the series of five experiments referred to above (p. 523), the only one in which a significant quantity of heat appeared to be produced after the end of relaxation.

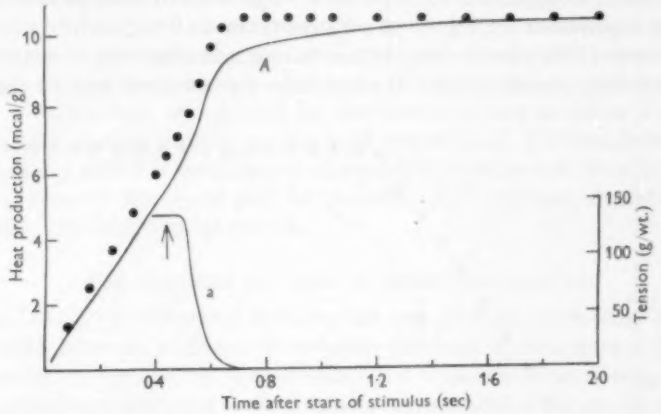

Fig. 7. Analysed deflexion (*A*) due to the heat produced in a 0·44 sec isometric tetanus of a pair of toad sartorii at 16·6° C; *a* later stages of tension. ●, deflexion after allowance for an assumed non-uniform distribution of the heat produced during contraction.

The average thickness of the muscles used in this experiment was $a = 0{\cdot}073$ cm, so $\alpha = k\pi^2/a^2 = 2{\cdot}3$. Assuming a non-uniform rate of heat production $u(1+\cos \pi x/a)$ ending at $t = 0{\cdot}64$ sec, the deficit of temperature at the thermopile before and after that time can be calculated from equations (5) and (6) respectively. The quantities so calculated are to be added to the ordinates of curve *A*, Fig. 7. If u be taken as 3·5 mcal/g.sec the corrected results are given by the filled circles. A curve drawn through these would reach a steady level by the time (0·72 sec) when the tension had fallen to zero, and the total heat after the last shock would be 4·2 instead of 5·1 mcal/g. The amount of heat in the non-uniform distribution is calculated as 3·5 mcal/g.sec continuing for 0·64 sec, i.e. 2·24 mcal/g, which is 21 % of the whole heat given out in the contraction. It is easy to admit that 21 % of the whole heat produced during contraction could be non-uniformly distributed: indeed if equation (9) had been used instead of equation (3), only about 17 % would have been needed.

A similar calculation was made for the other pair of toad sartorii referred to on p. 523, stimulated for 0·76 sec at 20° C. The observed heat after the last shock was 5·5 mcal/g and the temperature went on rising for

at least 1·0 sec after relaxation must have been complete. A non-uniform distribution of the heat production was assumed, as before, ending at 1·0 sec and amounting to 17% of the total heat in the contraction. The corrected temperature became constant at 1·1 sec instead of continuing beyond 1·8 sec, and the heat after the last shock became 4·8 mcal/g.

The most exaggerated example of a large delayed heat production is that in experiment (*e*), Fig. 3, of a 0·8 sec tetanus of frog sartorii at 17° C. The course of the rise of temperature during and after this contraction is shown in Fig. 8 (open circles). The heat after the last shock had the extreme

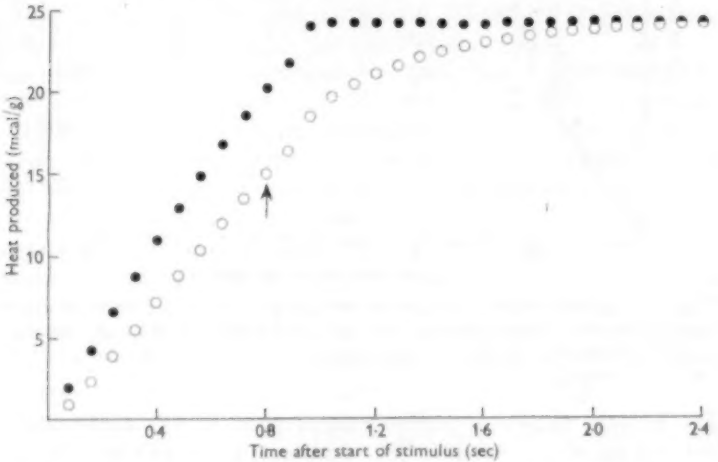

Fig. 8. An extreme example of presumed non-uniform distribution of the heat produced during a 0·8 sec tetanus of a pair of frog sartorii at 17° C. ○, heat deflexion after analysis in 0·04 sec time units; ●, the result of correcting for an assumed non-uniform distribution of the heat production. The last shock of the stimulus occurred at the arrow.

value of 9·1 mcal/g, and it continued for more than 1 sec after relaxation should have been complete at 1·0 sec. A noticeable thing about this experiment was that the heat between 0·8 and 1·0 sec was not excessive, in fact only 4·1 mcal/g, so that nearly all the excess appeared after relaxation was complete. This suggested that the non-uniform heat production was confined to the outside of the muscle, from where it would take time to reach the thermopile: so equation (9) was used instead of equation (3). The non-uniform heat production was assumed to end at 0·96 sec, i.e. 0·16 sec after the last shock, and with $\alpha = 2{\cdot}5$ the deficit of temperature was calculated every 0·08 sec up to 2·4 sec. This was then added to the ordinates of the curve of Fig. 8 (open circles) and the result

is shown by the filled circles. The heat required in the non-uniform distribution, in order to obtain the result shown, was 53% of the whole heat produced in the contraction. The effect of the calculation was striking, it transformed a curve taking about 1·6 sec after the last shock to reach its final level to one in which the process took only about 0·2 sec: while the total heat after the last shock became 4·0 mcal/g instead of 9·1 mcal/g. The only difficulty about accepting the result is that it required a large part of the whole heat to be produced in the outer half of the muscles: but the experiment was certainly abnormal. Possibly, by some mischance, a layer of inert fluid or material lay between the muscle fibres and the thermopile, though nothing of this kind was noticed. The result was not due to some casual error in the record analysed, because a number of other contractions of the same pair of muscles, with different durations of stimulus, provided similar records.

The relaxation heat after an isometric contraction

The 'hump' in the curve defining the rate of heat production during relaxation after an isometric contraction has been known since 1920. It occurs during the interval within which the tension is disappearing, so it was natural to attribute it to the degradation into heat of the elastic energy developed during contraction. In recent years methods were found of measuring this elastic energy (Hill, 1950*a*, *b*, 1953*a*; Jewell & Wilkie, 1958); and then it became apparent that the elastic energy was not large enough to account for the whole of the heat that appeared in the 'hump'. At about the same time, however, it was found (Hill, 1953*b*) that the release of tension itself, in a contracting muscle, causes a substantial production of heat (the 'thermoelastic' heat); and the amount of this was about sufficient, when added to the elastic energy, to provide the whole of the extra heat required. The thermoelastic heat was applied by Hill & Howarth (1959) to explain the otherwise anomalous results obtained in stretching muscles during contraction: and it was examined in more detail by Woledge (1961), who concluded that it is a reversible thermodynamic effect, which occurs in opposite directions when the tension rises or falls during the course of an isometric contraction.

The 'hump' therefore in the relaxation heat could be accounted for in terms of elastic energy and thermoelastic heat. But a considerable amount of heat appears after the last shock in an isometric contraction which cannot be attributed to these mechanical causes; presumably it is the 'activation heat' (Hill, 1949*b*) due to the last shock itself, together with the residue, if any, of the activation heat due to preceding shocks of the stimulus. In Figs. 1, 2 and 4 above interrupted lines have been drawn below the 'hump' to separate the elastic and thermoelastic energy (above)

from the residue of activation heat (below). These separating lines are somewhat arbitrary, but the idea on which they were drawn was:

(*a*) The activation heat would continue for a short time after the last shock at the same rate as before;

(*b*) it would then follow the same curve as the observed heat rate until the tension began to diminish;

(*c*) thereafter it would continue to fall on a smooth curve, until its rate became zero when relaxation was complete.

If the line separating the energy due to the fall of tension could be accurately drawn, the area below it would be a measure of the residue of the activation heat after the last shock.

In a frog or toad sartorius contracting isometrically the elastic energy in the muscle itself, due to the tension P developed in it, can be read off from Fig. 2 of a paper by Hill & Howarth (1959). If the muscle is connected to a tension recorder by a wire or thread, which is itself extensible,

TABLE 2. Mechanical components of the relaxation heat

Pl_0/M	Elastic energy, (g-cm/g)	Thermo-elastic heat, (g-cm/g)	Total, (g-cm/g)	Total, (mcal/g)
2000	17	28·0	45·0	1·06
2200	20·2	30·8	51·0	1·20
2400	23·8	33·6	57·4	1·35
2600	27·7	36·4	64·1	1·51
2800	31·8	39·2	71·0	1·67
3000	36·5	42·0	78·5	1·84
3200	40·7	44·8	85·5	2·01

the elastic energy developed in this also must be added. The elastic energy so calculated provides the first component of the relaxation heat. The thermoelastic heat produced when a tension P disappears, according to the latest measurements (Woledge, 1961), is 0·014 Pl_0/M g-cm/g. Table 2 gives the calculated values of the elastic energy in the muscle itself and the thermoelastic heat, for various values of Pl_0/M. The elastic energy developed in external extensible connexions is not included: that must be added separately.

Three experiments were made on toad sartorii at 0° C in order to obtain the greatest possible accuracy in attempting to separate the mechanical components of the relaxation heat from the residue of the activation heat. It was shown above that the errors due to a non-uniform heat production during contraction are much smaller if a muscle is slower, and the slowest available muscles were toad sartorii at 0° C. It was shown also that these errors are less in a thinner muscle: the muscles used were only 0·55 mm thick. They were stimulated isometrically for 3·3 sec with short condenser

discharges at 10/sec. The tension was recorded as well as the heat. In each experiment heat records were made of several contractions. These were measured every 0·1 sec and the results averaged. The mean was taken of the three averages. This was then analysed in time units of 0·1 sec, from 0·6 sec before the last shock to 2·0 sec after it. The rate of heat production is given at every 0·1 sec in Fig. 9 by the height of the open circles. The last shock was at time '0'. The tension is shown by curve (*a*): the mean value of Pl_0/M was 2270.

The interrupted line, an attempt to separate the two components of the heat produced after the last shock, is admittedly somewhat arbitrary, but in fact, bearing in mind the criteria referred to above, it could not be drawn very differently. The total heat produced after the last shock, up to a moment 2 sec later, was 2·70 mcal/g. The heat calculated from the area under the broken line was 1·13 mcal/g (though see the note below). The difference between these, namely 1·57 mcal/g, is the amount which, if the assumptions are correct, could be attributed jointly to elastic energy and thermoelastic heat. The calculated elastic energy included that in the wire to the tension recorder. The calculated total of elastic energy and thermoelastic heat came to 1·45 mcal/g. Considering all the assumptions and measurements involved, the agreement (between 1·57 and 1·45 mcal/g) is reasonably satisfactory.

Note. In Fig. 9 the rate of heat production given by the hollow circles appears to fall to zero rather sooner than the tension. This was probably caused by the early incidence of the negative delayed heat (Hill, 1961) which became apparent later. The error caused by this could not be large, because the rate of the negative heat production is small. Its effect would be to cause the interrupted line to be drawn rather too low near its end, so the residue of the activation heat may really be rather greater, say about 1·25 mcal/g instead of 1·13 mcal/g.

A similar calculation can be made with Fig. 2 above, in which the rate of heat production of toad sartorii at 16·6° C is shown, before and after the last shock of a 0·48 sec tetanus (25 shocks/sec). Here, also, as for Fig. 9, the numbers used for the analysis were the mean from three experiments; these were selected because the heat production did not continue beyond the end of relaxation. The total heat after the last shock was 3·6 mcal/g. The heat below the interrupted line after the last shock was 1·46 mcal/g, difference 2·14. The heat calculated from the tension for elastic energy and thermoelastic heat together was 1·81 mcal/g. The agreement again is fairly satisfactory. The mean value of Pl_0/M was 2750; the tension in a tetanic contraction is always rather higher at room temperature than at 0° C. This explains why the heat due to drop of tension is greater, and it may explain why the residue of the activation heat is greater too (since a muscle usually produces more heat when it develops a greater tension).

It is interesting that in Fig. 4, for frog muscles at 17·2° C, the area of the 'relaxation hump' above the interrupted line is 1·86 mcal/g, about the same value, even though quite a lot of heat appeared to be produced after the end of relaxation.

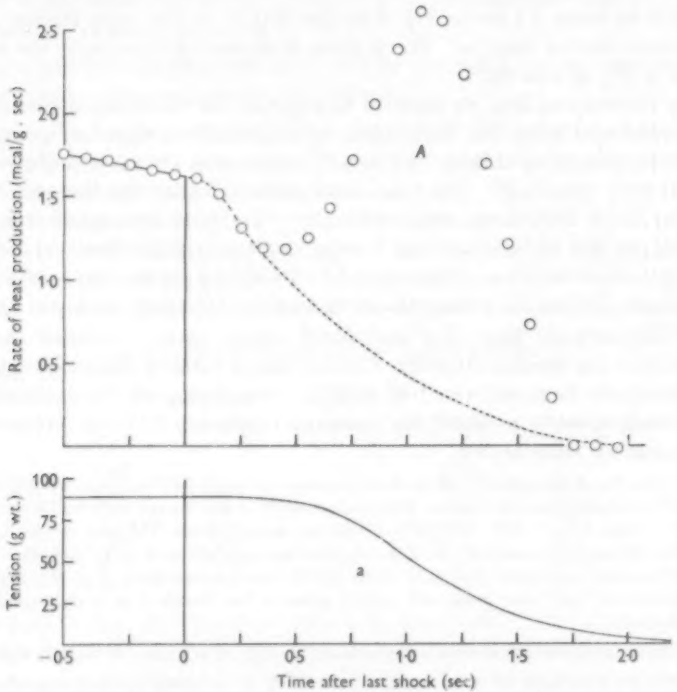

Fig. 9. Above: rate of heat production (*A*) before and after the last shock (at time 0) of a 3·3 sec isometric tetanus of toad sartorii at 0° C, analysed in 0·1 sec time units. Below: tension (*a*). The interrupted line under *A* gives the estimated rate of the residue of the activation heat after the last shock.

The residue of the activation heat after the last shock of a tetanus might be expected to be of the same order of size as the activation heat in a single twitch. The latter is not accurately known, but from Fig. 9 of a paper by Hill (1949*b*) it can be estimated in toad sartorii at 0° C as about 2 mcal/g: while in a later paper (Hill, 1950*b*) it was assessed as 'rather less than one half the heat given out with very small loads;' this leads to about the same quantity. There is approximate agreement therefore, in size, between the activation heat in a twitch and the residue of the activation heat after the last shock of a tetanus.

In frog muscles at room temperature the usual incidence of a non-uniform distribution of the rate of heat production during contraction prevents an accurate estimate from being made of the residue of the activation heat after the last shock of a tetanus. At 0° C, however, in two experiments with frog muscles (1 sec stimuli), the quantities obtained by the same method as was used in connexion with Figs. 2 and 9 were 2·21 and 1·74 mcal/g. In Hartree's experiment on frog sartorii shown in Fig. 1*A* the area below the interrupted line after the last shock was 0·207 of the whole initial heat, which was taken to be 7·5 mcal/g: so the residue of the activation heat would be 1·55 mcal/g. For frog sartorii, therefore, at 0° C an average value of 1·8 mcal/g is likely for the residue of the activation heat after the last shock. At 17° C the tension developed during a tetanic contraction is rather greater than at 0° C, so a rather higher value, say 2·0 mcal/g, is probable. With values of Pl_0/M of 2400 and 2800 respectively at 0 and 17° C, the heat due to the fall of tension would be 1·35 and 1·84 mcal/g: so the total heat after the last shock would be 3·15 and 3·84 mcal/g respectively at the two temperatures.

DISCUSSION

It appears therefore from the combined evidence, direct and indirect, presented above, that after an isometric contraction of frog or toad sartorii the rate of heat production falls to zero by the time that relaxation is complete; and that the total amount of heat produced after the last shock of a stimulus can be accounted for in terms of elastic energy, thermoelastic heat, and the residue of the heat of activation, or maintenance. When, in the present experiments, the heat production after the last shock appeared to be prolonged or excessive, the anomaly could be explained by assuming that the spatial distribution of the rate of heat production during contraction was non-uniform. In the older experiments of Hartree & Hill (1928) and Hartree (1932), made with instruments and methods much less reliable than present ones, various other causes may have contributed to the same effect, e.g. lack of flatness in the thermopiles, or errors due to the methods of 'control heating' and analysis. It must be noted that the results described in the present paper have no relation to the ordinary recovery heat, which develops gradually later.

The question, therefore, from which the present investigation started, of whether the early delayed heat production reported in the older papers could be a sign of the Lohmann reaction does not arise: for this heat appears to be due to error. If the Lohmann reaction really occurs during contraction and relaxation, its heat production, positive or negative, would be inextricably mixed with the rest of the heat produced by the muscle. If the Lohmann reaction occurs after the end of relaxation and before the

ordinary positive recovery heat production begins, its heat must be either zero, or small and negative (corresponding to the negative delayed heat, Hill, 1961). But in that case it remains a problem why chemical methods have been unable to detect any net hydrolysis of ATP at this time.

It is most desirable, therefore, that the net amount of heat produced, per mole, in the Lohmann reaction, in frog or toad sartorii, at 17° C and at 0° C, should be accurately known, under the conditions (of pH, etc.) believed to exist inside the fibres during life. If the heat really is positive it seems that the reaction must occur, if it does occur, during the time when the heat of activation, and its residue, are appearing. The early rapid decrease of pH during contraction, observed by Distèche following Dubuisson (see Distèche, 1960), was attributed by them to the hydrolysis of ATP, the later increase of pH to the splitting of phosphocreatine, with the resynthesis of ATP in the Lohmann reaction. Unless, however, the Lohmann reaction is thermally neutral the complete acceptance of this view is difficult, for the positive phase of pH change appears to continue after the muscle has relaxed, although no genuine positive heat production occurs until the ordinary recovery process begins. The delayed negative heat might have something to do with the later rise of pH, though it is not clear what. Can the heat of the Lohmann reaction really be negative? Or is it possible that in living muscle the Lohmann reaction is not a single affair, but a sequence of coupled processes? Such questions are unanswerable except by specific chemical methods. All that measurements of heat and pH change can do is to act as umpires in deciding what conclusions from chemical evidence are admissible on, or excluded by, the physical evidence, and perhaps to suggest where and how a solution should be sought.

The fact, certainly established by the present investigation, that the rate of heat production of a muscle may vary considerably from one region to another, has an important bearing on the technique of myothermic measurements. The conclusions from it are that, for real accuracy to be obtained in the resolution of rapid thermal events, (*a*) the slowest available muscles should be used (e.g. toad sartorii at 0° C) and (*b*) these should be as thin as the instruments available permit. The time scale of the redistribution of temperature in the muscles can thus be reduced in comparison with that of the physiological changes themselves. This has long been known in a general way, but the extent of the errors involved was never explicitly realized until the present analysis was made. In following slower events, e.g. those of aerobic or anaerobic recovery, the same technical problem does not arise; for (except in very thick muscles) thermal equalization is usually rapid enough to forestall any serious trouble arising from non-uniform distribution of the heat. When indeed rapidity of heat

loss is a dominant factor in limiting accuracy of measurement there is even an advantage in using larger muscles, in which the rate of temperature loss is less; or in working at a higher temperature where the process investigated takes less time.

The fact that the residue of the heat of activation that appears after the last shock of a tetanus is about the same as the heat of activation in a single twitch, provides an interesting addition to what is known about the 'active state'. It was realized long ago (Hill, 1913) that the heat produced by a muscle under a tetanic stimulus of given duration increases at first with the frequency of the shocks constituting the stimulus; but becomes constant above the comparatively low frequency which produces a complete, or nearly complete, summation in the mechanical response. This is not because some of the shocks fail to excite, but because the individual thermal response to each shock becomes successively less as the frequency rises. It seems that the active state (Hill, 1949*d*) is raised to its maximum level by each shock in turn, so with a high enough frequency it continues practically at its full height. The duration of the active state in a twitch can be considerably extended by exposing a muscle to a solution containing nitrate or other anions in place of the usual chloride: and the heat produced in the twitch is then increased (Hill & Macpherson, 1954). But during the tetanic contraction of a muscle so treated the heat production remains what it was before treatment: only the residue of the activation heat after the last shock is increased.

It appears, therefore, that during a tetanic contraction the intensity of the active state is maintained at the full level which is momentarily reached during a twitch. Each successive element of the stimulus brings the active state back to its full intensity. If so, it could be expected that the residue of the heat of activation after the last shock of a tetanus would be independent of the duration of the stimulus (as it appears to be) and the same as the heat of activation after a single maximal shock. This does not explain what the active state is, but adds another characteristic which may help in its elucidation.

It was shown above that the amount of heat in the 'hump' that appears during relaxation from an isometric contraction can be accounted for fairly accurately in terms of elastic energy and thermoelastic heat. Experimentally there can be no doubt that this 'hump' always occurs during the interval in which the tension is diminishing. Some of it must certainly be due to the elastic energy which disappears when the tension falls, but it is impossible to explain the whole of it in this way. The thermoelastic heat production was first observed during the rapid experimental release of tension in a contracting muscle; but it was open to question whether the same effect would occur when the tension fell naturally during

relaxation. The fact that the missing heat can be attributed, with fair accuracy, to the thermoelastic effect of falling tension, helps to remove that doubt; particularly when one remembers that the constant 0·014 in the expression 0·014 Pl_0/M for the thermoelastic heat was not chosen for the purpose but obtained experimentally (Woledge, 1961). The result, in conjunction with Woledge's evidence that the thermoelastic heat really is a reversible thermodynamic effect, gives one the greater confidence in applying the concept of thermoelastic heat without reservation.

One such application that now seems justifiable is to the earlier stages of the heat production during an isometric, or an after-loaded isotonic, contraction, while the tension is rising. This application was in fact made by Hill & Howarth (1959, pp. 185, etc.), but only for a rather specific purpose. It deserves a more general statement. A rise of tension should cause an absorption of heat, so the observed heat production would be correspondingly too small. In considering the chemical mechanism of contraction the liberation of energy that matters is that due to chemical processes, the purely physical effect due to change of tension is of no direct concern. Moreover, during the time while the tension is rising, mechanical work is being done in stretching the elastic elements of the muscle. The energy therefore which is really being liberated by a muscle during the early phase of contraction is considerably greater than that which appears as heat. This makes a considerable difference: indeed, during the interval in which the tension is rising the extra energy supplied by chemical processes is equal to the area of the 'hump' during relaxation, which is of the order of 1·5–2 mcal/g. The subject will be examined further by Woledge in a later paper.

Strong evidence, direct and indirect, was presented above that the heat produced in contracting sartorii is not uniformly distributed. This is not a random affair; nearly always it appeared that the rise of temperature was greater in the 'inside' region of the muscles than in the 'outside' region, the 'outside' being the part which in the intact animal is next to the skin, and in myothermic experiments is normally in contact with the thermopile. The amount of heat produced in a tetanic contraction of given duration depends on the speed of the muscle, a faster muscle giving more. The quicker muscle of a frog produces more heat than the slower muscle of a toad, much more than the very slow muscle of a tortoise; while a rise of temperature, which increases the speed of any muscle, causes it also to produce heat during a tetanic contraction at a higher rate. A natural explanation, therefore, of the difference found between different parts of a sartorius would be that the fibres in the 'inside' region are faster than those near the 'outside'.

No way of testing this expectation experimentally was evident, but it

is known (Hill, 1949*c*, p. 234) that the fibres near the 'outside' tend to be smaller than those elsewhere. It is known also (Cattell, 1928) that the fibres of the frog sartorius are innervated from two roots, the VIIth and VIIIth, a certain number of them being supplied from both. Moreover it was shown by Buller, Eccles & Eccles (1960) that the speed of a muscle can be altered by transferring its innervation from a 'slow' to a 'fast' motoneurone. It is possible, therefore, that the fibres innervated separately from the VIIth and VIIIth roots would differ in speed, the faster ones therefore producing more heat during a tetanus. If the innervation were regional, the non-uniformity of the heat production would be explained.

SUMMARY

1. Older measurements, now believed to be erroneous, of the heat produced by a muscle soon after the end of relaxation, had suggested that this heat might be related to chemical reactions supposed to occur during a short interval after contraction.

2. Experiments are described in which the heat produced, by frog or toad sartorii, after the last shock of an isometric tetanus, was measured, both at 0° C and at room temperature.

3. At 0° C the heat production ended by the time when the tension had fallen to zero: and its total amount could be explained in terms of known quantities.

4. At room temperature (around 17° C) very variable results were obtained. The heat apparently produced after the last shock generally lasted beyond the end of relaxation and might be considerably greater than could be explained in known terms. These effects were much more obvious in the faster muscles of frogs than in the slower muscles of toads.

5. A mathematical and numerical analysis showed that all this variation could be explained by assuming that the spatial distribution of the heat produced during contraction was not uniform. It showed also that in slower muscles, in which the rate of heat production was less and the duration of relaxation greater, thermal equalization would be rapid enough to reduce these anomalies; while at 0° C, where muscular processes are very slow, it would obviate them entirely.

6. A direct comparison of heat records with single muscles, arranged first one way round on a thermopile and then the other way, showed that these irregularities in the distribution of the heat produced during contraction do in fact occur.

7. It is concluded that at room temperature the real heat production, after the end of a stimulus, ends, as it does at 0° C, by the time that the mechanical response has disappeared. After that, slower processes come

on, first the small negative delayed heat and then the much larger recovery heat.

8. The residue of the heat of activation (or maintenance) after the last shock of a tetanus was examined. It is about the same as the heat of activation in a single twitch. The reason for this is discussed.

9. The 'hump' in the relaxation heat after an isometric contraction can be fully accounted for in terms of the elastic energy developed during the previous contraction and degraded into heat during relaxation, together with the thermoelastic heat due to the fall of tension.

10. These thermal effects of the disappearance of tension, when added to the residue of the heat of activation, can account for the whole of the heat produced after the last shock of a stimulus.

11. The cause is discussed of the irregularities in the distribution of the heat produced by a muscle during contraction.

12. The conditions necessary for accuracy in the resolution of rapid thermal changes during contraction are considered. The slowest and thinnest muscles are the best.

13. The need is emphasized of accurate information about the heat of reaction of chemical processes supposed to occur in muscular contraction, under the actual conditions believed to exist inside living muscle fibres.

My warm thanks are due to Mr R. C. Woledge for his skilled assistance in much of the experimental work described in this paper, and for making some of the critical experiments himself. He and I are indebted to the Medical Research Council for granting him a scholarship for training in research.

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THE INITIATION OF CUTANEOUS NERVE IMPULSES IN ELASMOBRANCH FISHES

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The majority of vertebrate sensory nerves have fine, branched, non-myelinated endings, but it is by no means certain what events occur in these fine branches. For example, in some sense organs the site at which the propagated action potential originates is not at the extreme end of the fibre but is more proximal, for instance at the first node (Gray, 1959; Murray, 1956; Paintal, 1959). On the other hand, in frog muscle stretch receptors (Katz, 1950), the small action potentials which were recorded from the axon some distance away were believed to have arisen in the terminal branches of the fibre, and the presence of propagated potentials in the terminal branches of the cutaneous nerves of the frog has been postulated on indirect evidence (Catton, 1958).

The present experiments involve a new and simple technique for recording the impulses in single cutaneous nerve fibres of elasmobranch fishes from outside the fish and without dissection. The results provide further evidence concerning the general problem of the mechanism and site of impulse initiation in sense organs.

The anatomical details of the innervation have also been briefly studied, for the earlier description of free nerve endings in elasmobranch skin (Wunderer, 1908) is inadequate because it does not describe the course of the fibres relative to the stratum compactum of the dermis.

METHODS

Various common *Raja* species were employed (*R. clavata*, *R. brachyura*, *R. montagui* and *R. naevus*); the results from all were similar. Some dogfish (*Scyliorhinus canicula*) were also used.

Electrical recordings were made with conventional a.c. amplification, having a time constant of 10 msec or longer.

Controlled mechanical stimuli were provided by a probe attached to the cone of a modified mains-energized loudspeaker, since greater movements were required than could be obtained with a crystal. The movements were damped by covering the face of the speaker with a metal plate, and by packing the back of the cone with foam plastic. The time constant of the movement was 1 msec, a rise time as short as this being obtained by augmenting the start of the energizing current with the discharge from a 500 μF condenser. The speed of movement of

the stimulator was therefore slow compared with the movements of a crystal stimulator, but was fast enough compared with the latencies obtained from the preparation. The stimulator could be triggered by hand or by relay from an electronic pulse generator, so that intensity, duration and repetition rate could be conveniently controlled.

For galvanic polarization a constant-current stimulator was used, with which small currents in either direction could be smoothly and slowly altered (the circuit was described by Lowenstein, 1955).

The temperature of the skin was changed by bringing close to it a hot glass rod, an electrically-heated coil or a metal container cooled with solid CO_2. Slower changes of temperature were obtained with a 12 mm cube of semiconductor materials held 1–2 mm from the skin; temperature gradients in either direction were established in this 'inverse thermopile' by the thermo-electric effect of an applied current. Skin temperatures were monitored with a fine thermocouple junction resting in the mucous layer covering the skin.

In the anatomical investigation axons were stained with methylene blue and followed in whole mounts, and myelin was seen in sections of skin which had been treated with fixatives containing osmium tetroxide.

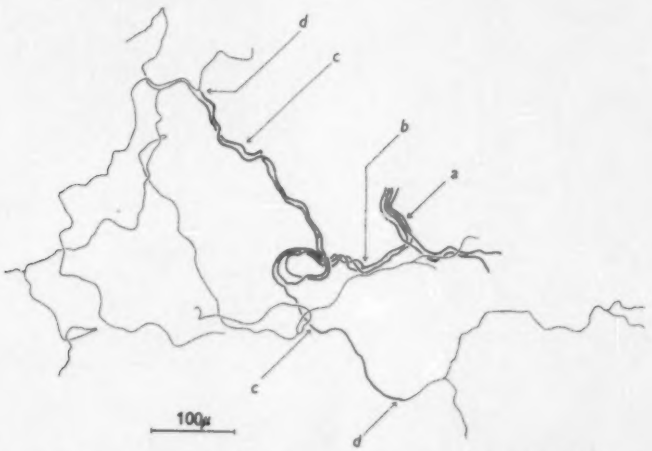

Fig. 1. The terminal ramifications of two cutaneous nerve fibres in the skin about 2 mm anterior to the upper lip of *Raja clavata*, drawn from a whole-mount stained with methylene blue. *a*, part of subdermal plexus; *b*, point of branching, resulting in two fibres which reach the surface in separate channels through the stratum compactum; *c*, where the fibres pass up through the stratum compactum; *d*, estimated end of myelination.

RESULTS

Anatomical

In whole mounts the dermal plexus can be clearly seen. At intervals, small bundles of one to eight fibres branch off and run outwards in channels through the stratum compactum (Figs. 1, 2). The fibres are 3–6 μ in diameter, and the channels through the stratum compactum are about 1 mm apart in the upper-lip region of a large fish; the fibres are usually

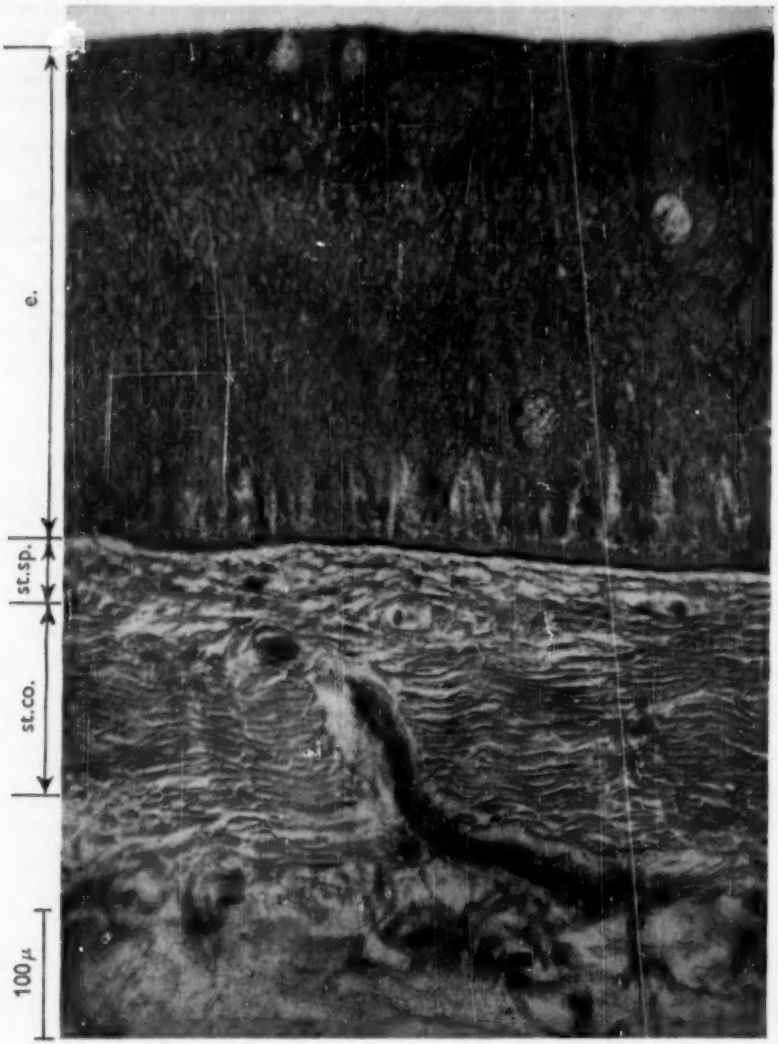

Fig. 2. Transverse section through the skin of *R. clavata* about 2 mm anterior to the upper lip, showing the extent of the epidermis and upper layers of the dermis, and the course of the myelinated fibres through the stratum compactum (fixation with osmium tetroxide, otherwise unstained). e., epidermis; st.sp., stratum spongiosum; st.co., stratum compactum. Scale, 100 μ.

accompanied by a small artery and vein. The fibres then run laterally in the stratum spongiosum for several hundred microns before ending there or in the epidermis; the exact location of the endings has not been studied. The myelination ends about 100 μ distal to the point of emergence of the fibres from the stratum compactum. The possible effect of fibre branching on the recorded potentials is discussed later, and so it is important to know to what extent it occurs. As can be seen in Fig. 1, the non-myelinated terminals branch extensively, but these divisions are all distal to the stratum compactum. There are also divisions of the myelinated fibres in the subdermal plexus, and the resulting branches usually run through different channels in the stratum compactum. The thickness of the different layers in upper lip skin (Fig. 2) is typically: epidermis, 350 μ; stratum spongiosum, 50 μ; stratum compactum, 150 μ. The corresponding dimensions for the skin over the gill bar are, for example, epidermis 100 μ, stratum spongiosum virtually absent, and stratum compactum 100 μ.

Occurrence of the cutaneous action potentials

If a pithed ray is laid ventral side up with an indifferent electrode making contact somewhere with the body, diphasic action potentials can upon application of a suitable stimulus be recorded with a wire electrode gently touching the surface of the skin (Figs. 3, 4). The wire need not be particularly fine (0·1–0·2 mm diameter) and the potentials can be recorded from almost any part of the skin where it is not too thick. The ventral surface of the snout gives the best results, especially on the flaps of skin beside the nares and on the fold of the upper lip, but similar potentials have been recorded from the rest of the ventral surface, dorsally from the margin of the spiracle and from the pharyngeal epithelium, especially from the gill bars. In *Scyliorhinus* the denticles make recording from the outside skin impossible, but action potentials can be recorded from the pharyngeal epithelium, again most clearly from the gill bars. Single units can be identified by their constancy of spike size and regularity of discharge, located at 0·25–2 mm spacings, agreeing with the density of innervation found anatomically. For example, on the upper lip of a small ray (span 15 cm) it is almost impossible to isolate a single unit, but this can easily be done in a bigger fish (span 40 cm) where the separate units are 1 mm apart. Further forward on the snout of a big fish the units were detected at spacings of about 2 mm.

The recorded size of the potentials varies with the extent to which the skin has dried. The mucous covering retards desiccation of the skin so that at room temperatures below about 18° C recording is possible for at least 4–5 hr after the fish has been taken out of the water and killed; the larger the fish, the longer the preparation lasts. As the mucus dries, the size of

the potentials increases, and values of 100–150 μV have been obtained (Fig. 4). Wetting the skin reduces the size of the recorded potentials, and will normally make them too small to be distinguished on the oscilloscope above the noise level of the amplifier, although they may still be heard in the loudspeaker. The skin covering the gill bar is more delicate than that on the surface of rays and dries up more rapidly; gill preparations were

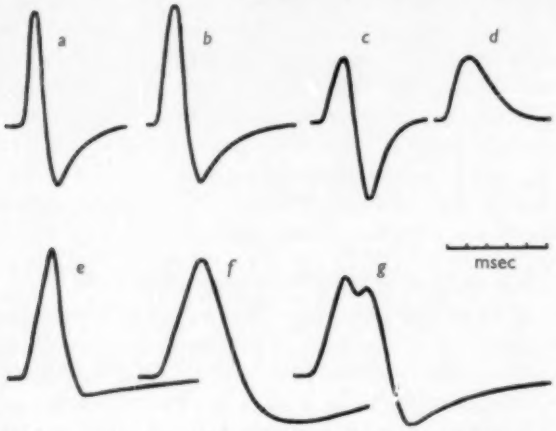

Fig. 3. Action potentials recorded from the skin of rays showing some of the various shapes and durations which are found. Deflexion is upwards when the recording electrode is positive relative to the indifferent electrode.

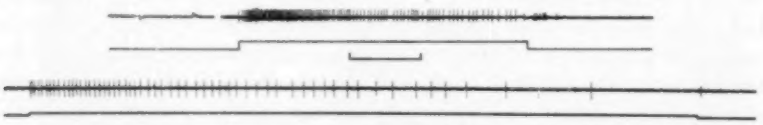

Fig. 4. The response of two separate cutaneous fibres to maintained mechanical stimulation, showing their rapid adaptation. In each record the lower trace marks the duration of the stimulus. Time scale, 1 sec. Voltage calibration for upper record, 50 μV.

therefore normally kept under liquid paraffin. Treatment with 2% novocain abolished the potentials, the test being made after an interval to allow for suitable drying.

The potentials occur in response to mechanical, thermal, chemical, and electrical stimulation; however, chemical stimulation is only possible by means of vapours such as ammonia or hydrochloric acid, since aqueous solutions prevent recording. Mechanical stimuli were applied either by touching the skin alongside the recording electrode—a nylon filament exerting a force of less than 5 mg wt. or a movement of about 20 μ with a

rigid probe were adequate—or more easily by altering the pressure of the recording electrode itself. In practice, when searching for suitable units for recording the act of touching the skin with the electrode provided the necessary stimulus.

The individual action potentials

Polarity. The first phase of the potentials is always positive, recorded at the electrode resting on the skin over the stimulated nerve ending. In only one instance out of several hundred records was the first phase negative. The polarity was checked by direct comparison with calibration voltages derived simply from a battery, and did not depend on the advertised polarity of a ready-made calibrator.

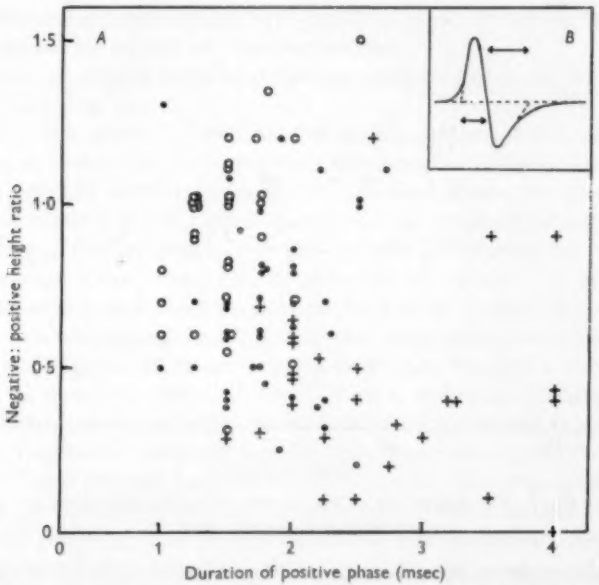

Fig. 5. *A.* The duration and proportions of the recorded potentials; the ratio of heights of the negative and positive phases is plotted against the duration of the positive phase. The symbols refer to the duration of the negative phase: ○, < 2·5 msec; ●, > 2·5 < 4·5 msec; +, > 4·5 msec. *B.* Method of measuring the duration of the positive and negative phases.

Relative amplitude of positive and negative phases. The relative amplitude of the positive and negative phases varies considerably. A few units are completely monophasic, and in a few the second phase is larger than the first; Fig. 5 shows the spread in a sample of 100 units. From this it can be

seen that there is a slight tendency for short-duration spikes to be more nearly diphasic than the longer ones. There is also a tendency for potentials recorded soon after the death of the fish to be more nearly diphasic than those recorded later. Because the shape of recorded potentials may be distorted by the amplifying system, a time constant of 1 sec was at times used. There was no difference in the shape of the recorded potentials.

Duration. The potentials are considerably longer in duration than those recorded from the same nerve fibres on their way to the C.N.S. (Fig. 13). In the sample of 100 units, the mean duration of the positive phase was 1·9 msec, with a range from 1 to 4 msec (Fig. 5); the negative phase is approximately twice as long as the positive, but there is much variation.

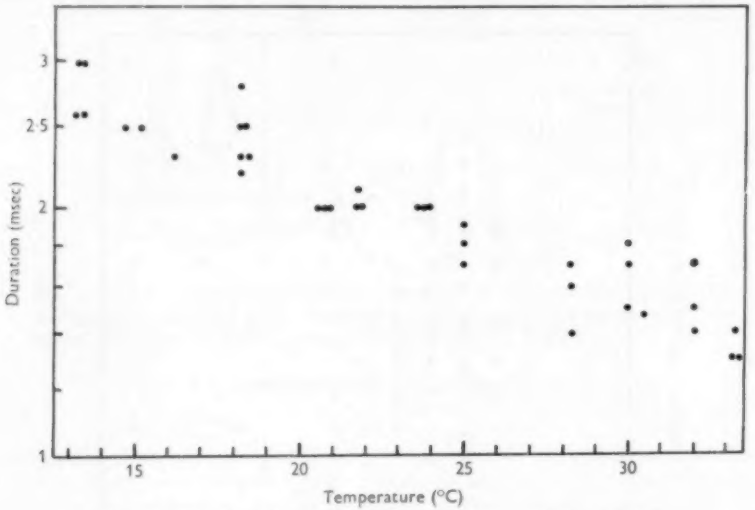

Fig. 6. The dependence of the duration of the positive phase on temperature in a single unit.

These values refer to records made at temperatures of 16–20° C; at higher temperatures the potentials are shorter, and at low temperatures longer (Fig. 6). In eight units studied in detail, the Q_{10} for the duration ranged from 1·2 to 2·3, with a mean of 1·6. The amplitude of the potentials, too, was reduced on warming.

Double spikes. In a few records a double peak was found to either the positive or the negative phase (Fig. 3*g*). There are two possible relationships of the double potentials to the mechanical stimuli which give rise to them: either they are the first, and often the only response to the onset of a light touch, or else they occur consistently, forming all the potentials of

the repetitive response to a maintained touch. There is evidence that in the former instance the potentials are compounded of two or more separate, simple, diphasic potentials; on occasion, by reduction of stimulus intensity, the second peak is lost and the amplitude of the potential is reduced in a quantal manner. Such quantal changes in amplitude also occur even when the compound potential has a simple shape without a double peak. When the point of stimulation is changed, the shape of the compound potential is also altered; for example, a change from a stimulus position 1 mm away from the recording electrode to pressure with the recording electrode itself resulted in a change from a double-peaked potential to one of simple diphasic shape but of greater amplitude and shorter duration.

On the other hand, when the double-peaked potentials occur repetitively during maintained stimulation, they are all-or-none. However, their shape can be altered by change of stimulus position.

Triphasic potentials occur at occasional recording sites, especially in gill preparations (Fig. 11).

Latency. The latency of initiation of an impulse on sudden mechanical stimulation varies with the strength of the stimulus and with temperature (Fig. 7). As the stimulus intensity is increased above threshold so the latency is reduced, but the curve flattens out at a certain minimum value irrespective of further increases in the strength of the stimulus. There are therefore two values which can be given for the latency of any unit, a maximum at threshold, and a minimum; both show considerable variation; minimum values ranging from 2·2 to 20 msec with a median value of about 5 msec, and maximum values ranging from 6 to 70 msec with a median value of 15 msec (measured at 16–20° C in a sample of 23 units). In a further 20 units the full intensity–latency relationship was not obtained, and single values at intermediate stimulus intensities ranged from 2·7 to 16 msec. These intermediate stimulus intensities were employed because at threshold the latency became variable, and so was unsuited for experimentation, while strong stimuli could not be used repeatedly without the preparation changing in sensitivity.

The effect of temperature on the intensity–latency curve illustrated in Fig. 7 is typical in that there is a general shift of the curve downwards on warming. But the comparison of latencies at different temperatures is not always as simple as in this unit, for there is commonly a change of sensitivity of the unit with change of temperature. This change of sensitivity may be in either direction, and means that a certain value of stimulus strength is not equivalent at each temperature, and therefore the latency will not be equivalent (since latency depends on stimulus intensity). Unless it can be seen that the curves lie below one another, and are not

displaced laterally, the comparisons of latency at different temperatures must be restricted to the minimum and threshold values. The relationship between latency and temperature in a number of preparations is illustrated by Fig. 8; the mean value for the temperature coefficient (Q_{10}) is 2·1.

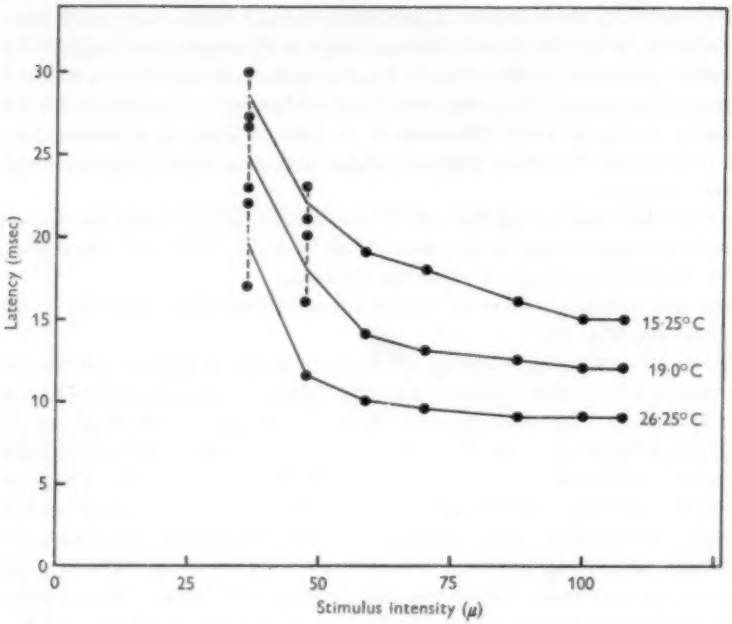

Fig. 7. The relationship between latency of impulse initiation following a mechanical stimulus, and the strength of that stimulus, recorded at three different temperatures. Near threshold the latency was variable.

Stimuli have been applied which are considerably shorter in duration than the latency. When this is done the latency remains the same, whatever the stimulus duration, unless the latter is so short that the mechanical stimulator has insufficient time to reach its maximum movement, i.e. less than 2 msec. Thus, for example, in one preparation a latency of 5·5 msec followed the onset of mechanical stimuli of both 2 and 10 msec duration, and in another a latency of 18 msec followed a stimulus of 6 msec duration.

The absence of intermediate minimum latencies of about 7 msec (at 18° C), which Fig. 8 illustrates, is found in the whole sample of 40 units whose latencies were measured, but it is not yet possible to say what significance, if any, should be attached to it.

In general, gill preparations have shorter latencies than have skin

preparations, their minimum values ranging from 2 to 5 msec with a mean of 3·8 msec (8 units).

'*Refractoriness*'. The preparation exhibits 'refractoriness' of considerable duration. This can be shown in two ways: qualitatively, when, for example, a test stimulus is applied at various intervals after 10 or 15 sec maintained

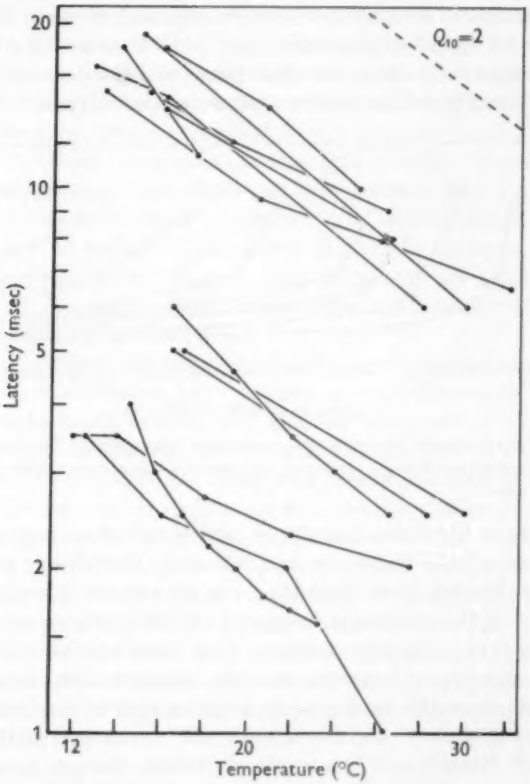

Fig. 8. The dependence of latency on temperature. The dotted line in the upper right corner has been drawn with a slope corresponding to $Q_{10} = 2$.

pressure, the interval has to be 2–5 sec before the response to the test stimulus is as great as to the onset of the preceding maintained pressure. Quantitatively, it is easier to work with regularly repeated stimuli, and to observe the latencies of impulse initiation at different frequencies and intensities of stimulation. Since for single stimuli latency varies with intensity, the changes in latency afford a sensitive indicator of the changes

in effectiveness of the stimulus. As the frequency increases so also does the latency, until at a sufficiently high frequency the response is lost altogether. But the latency can be reduced again by increasing the intensity. Thus the results can be brought into line with conventional representations of refractoriness, and the strength of stimulus required to maintain a certain latency (i.e. an effectively constant stimulus) can be plotted against frequency (i.e. interval between adjacent stimuli) as in Fig. 9. A true value for the absolute 'refractory' period cannot be given, for if a very strong stimulus is used, the subsequent responses are not reliable. It can be seen from Fig. 9 that relative 'refractoriness' extends to frequencies

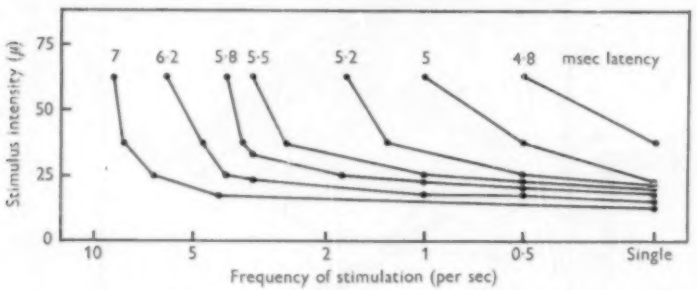

Fig. 9. 'Refractoriness' to repetitive mechanical stimulation. The stimulus intensities required to maintain the latency at constant values are plotted against the repetition rate.

of at least one every 2 sec, and this is typical. At the onset of repetitive stimulation at a fixed frequency and intensity, the latency progressively increases over the first 5 sec from the value for a single stimulus up to that characteristic of the particular frequency and intensity selected.

Preliminary experiments indicate that 'refractoriness' is scarcely affected by changes of temperature; this measurement is not, however, easy to make, since the most readily detected sign of the 'refractoriness' is the change of latency, and the latency will be changed at the new temperature both directly and as a result of possible changes in sensitivity of the unit. Thus changes in 'refractoriness' with temperature can only be assessed if the intensity-latency relationship and the absolute sensitivity of the preparation are found for each temperature. Few preparations remained unaltered in sensitivity as a result of the long series of mechanical stimuli which such testing involved.

As a qualitative test, at a number of temperatures, the stimulus intensity was set at a point about half way between threshold and the lowest value for minimum latency; for example, if the preparation illustrated in Fig. 7 had been used, a value of about 50 μ would have been chosen for the

intensity. The frequency of stimulation was then increased, and at a certain point the latency was found also to start increasing, indicating the onset of relative 'refractoriness'; this frequency was found to be unaffected by changes of temperature.

Antidromic stimulation

In the gill preparations (usually of *Scyliorhinus*) the sensory nerves running from the skin of the pharyngeal face of the gill bar are readily accessible (Fig. 10*A*) and so antidromic impulses can be initiated in the cutaneous fibres by electrical stimulation of the exposed nerve; these antidromic impulses can then be recorded by the electrode on the skin surface (Fig. 10*B*). A direct comparison is therefore possible between impulses initiated by mechanical stimulation at the electrode and the antidromic impulses. The former will here be referred to as orthodromic although this description may be technically incorrect. Figure 11 shows an example of such a comparison; it can be seen that the shapes are similar and that the polarity is the same. Both orthodromic and antidromic impulses are recorded with the positive phase first.

In these experiments the pre-trematic branch of the nerve which runs to the posterior face of the gill bar was used because it contains fewer fibres than the post-trematic branch, and none of them are motor (Goodrich, 1930). Certainly no muscle activity was seen when the nerve was stimulated.

The antidromic impulses are most easily recorded, and are largest, at sites where orthodromic impulses are also readily obtained, namely the ends of the large papillae (Fig. 10*B*), and the row of small lumps which lie between them. Over the course of the nerve abreast these recording sites, the antidromic impulse was much smaller and it could barely be detected by an electrode touching the nerve itself where it emerged from the gill tissues into the dissection cut. The recorded potentials could not be some kind of stimulus artifact, as the stimulus was only 50 or 150 μsec long, and the recorded potential followed it with a latency (depending on the distance) of 1–2·5 msec.

Simultaneous nerve and skin recording

With the gill preparation described in the last section it is possible to record not only from the surface of the skin but also from the branchial nerve, which can be thinned down by dissection until only a few intact fibres are left; the region of skin which is served by these fibres can then be identified, and when this region is touched by an electrode connected to a second recording channel and stimulated mechanically, impulses are recorded in both channels with a 1:1 relationship between them (Figs. 12, 13). The two recorded potentials have distinct shapes and durations, the

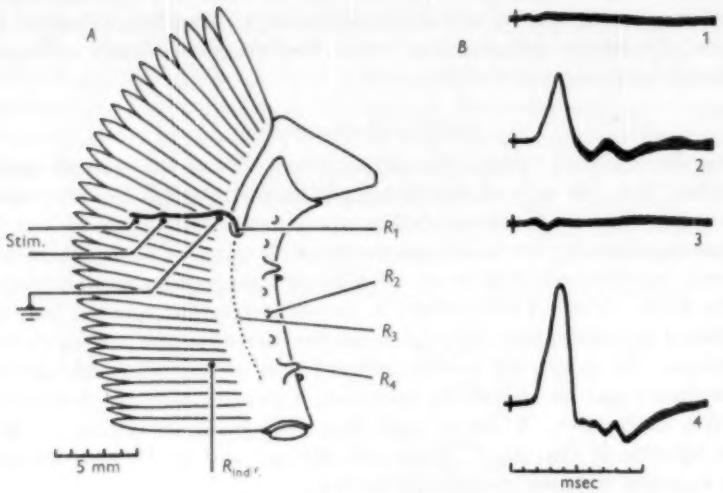

Fig. 10. Antidromic stimulation of gill preparations. *A*. Anatomical diagram indicating the method of recording and the sites (R_{1-4}) from which the records in *B* were obtained. Stim., stimulating electrodes on the branchial nerve; $R_{indif.}$, the indifferent recording electrode. *B*. Antidromic activity at four sites on the gill bar when the nerve was stimulated electrically. Deflexion upwards represents the recording electrode positive to the indifferent electrode. The stimulus artifact is indicated at the beginning of each trace.

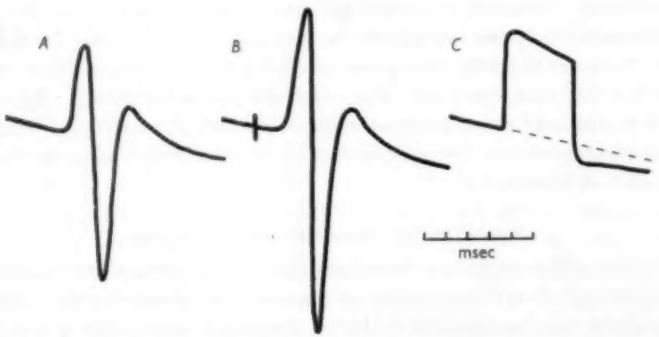

Fig. 11. Potentials recorded from the same site on the surface of the gill bar. *A*. Mechanical stimulation of the skin; *B*. Electrical stimulation of the branchial nerve; *C*. 25 μV rectangular calibration pulse.

potential from the skin lasting much longer than the nerve potential. The skin potential starts before the other, usually by less than 1 msec, although the slow rise of its positive phase makes the exact timing difficult to determine (Fig. 13). This time interval of less than 1 msec may be compared with the 1–2·5 msec required for antidromic propagation over the same distance, as described on p. 557. A possible explanation of the difference in the

Fig. 12. Action potentials recorded simultaneously from the branchial nerve (upper trace) and from the surface of the gill bar (lower trace) during mechanical stimulation near the latter site. Time scale, 0·1 sec. The record runs continuously from top left to bottom right.

two time intervals will be discussed later, but since it may depend on the detailed spatial relationship of stimulating and recording sites, it may be noted that in the experiment illustrated in Fig. 12 the mechanical stimulus was applied at the base of one of the papillae and the recording electrode was at the tip, i.e. 1·5 mm away.

Even when the simultaneous double recording was not obtained, it was clear that the latency and 'refractoriness' of the action potentials recorded from the pre-trematic branchial nerves on mechanical stimulation of the gill bar were similar to those of the potentials recorded directly from the surface.

Galvanic polarization of the skin

Additional evidence about the site of impulse origin can be obtained from experiments in which the process of impulse initiation is affected by a direct current passed into the skin from an electrode close alongside or

surrounding the recording electrode; the preferable method of using the recording electrode itself for stimulating was not satisfactory in this case because the current strength was such that the recording became noisy even with non-polarizing Ag/AgCl wire. The results were not entirely clear-cut, but of those units in which a definite change of impulse discharge frequency could be identified on altering the current, 60 out of a total of 75 units were excited by an anode on the skin and inhibited by a cathode.

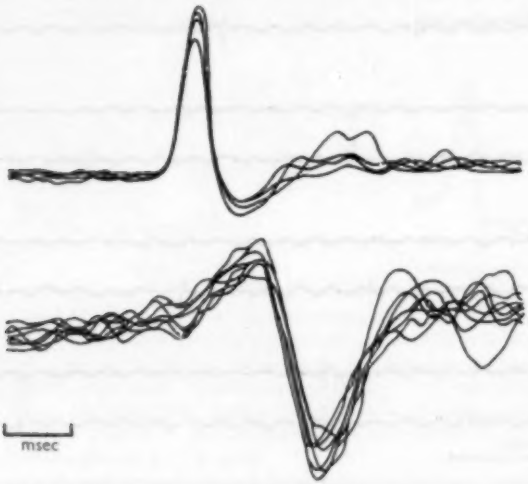

Fig. 13. Action potentials recorded at the same time from the branchial nerve (upper trace) and from the surface of the gill bar (lower trace) on mechanical stimulation. The records have been superimposed so that the branchial nerve potentials coincide.

In some units the shape of the recorded potentials could be clearly seen to be altered by the current. In all these it was surface anodal which was excitatory and this was accompanied by a decrease in the height of the positive phase and an increase in the negative.

Cutaneous nerves as sense organs

The cutaneous nerves were found to have the type of sensitivity expected of free nerve endings in lower vertebrates and therefore this aspect of their function was not studied in detail.

The sensitivity of the response to mechanical stimulation has already been mentioned (p. 550). In response to such stimulation a rapidly adapting train of impulses is evoked (Fig. 4); the time for adaptation to be three-quarters completed ranges from 1 to 5 sec. In preparations several hours

old the adaptation may not be complete, and an irregular 'resting' discharge continues at a low frequency all the time the recording electrode is touching the skin. It is of course impossible to record from skin without stimulating it and so the question of spontaneity cannot be discussed.

Responses to both warming and cooling have been obtained—that is, some units fire faster when warmed and slower when cooled, and others faster when cooled and slower when warmed (Fig. 14). It is only possible to detect decreases in frequency when there is incomplete adaptation to maintained mechanical stimulation, but an assessment can be made by observing the change in the transient response to a standard mechanical stimulus applied during the warming or cooling process.

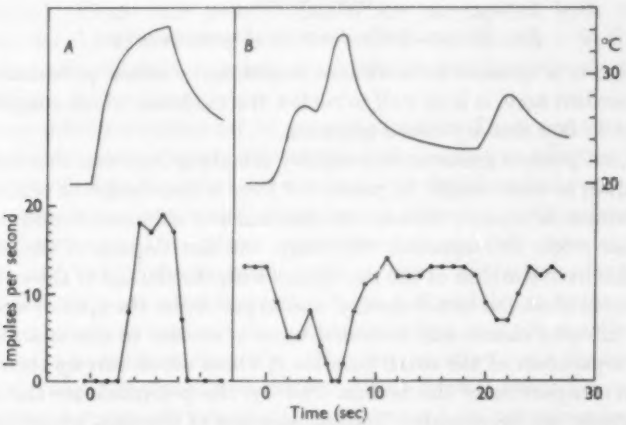

Fig. 14. The response of cutaneous fibres to thermal stimulation. *A*, a 'warm' fibre; *B*, a 'cold' fibre. Lower trace, impulse frequency; upper trace, temperature. The resting activity in *B* was 'spontaneous' in that there was no stimulation other than the contact with the recording electrode.

If the change of temperature is relatively slow, i.e. less than 1° C/sec, then warming from room temperature (19–20° C) to about 30° C initiated continuous activity; but if the rate of change was more rapid (more than 1·5° C/sec) then the threshold was 2–3° C above room temperature.

The greatest cold sensitivity was found in one unit which had the usual response to a standard mechanical stimulus of a brief burst of two to three impulses; it ceased to respond when warmed from 20 to 20·8° C in 2 sec, but when the temperature remained steadily at 20·8° C the response returned.

'Hot fibre' spikes, initiated by temperatures about 30° C, were small, and 'cold fibre' spikes were large. This does not, however, necessarily

indicate a difference in the fibre size because of the direct effect of temperature on spike amplitude.

Chemical stimulation was applied by holding a drop of concentrated ammonia solution close to the skin (the temperature of the skin did not alter by more than 0·5° C). In most preparations after about 15 sec a non-adapting discharge took place, with impulses similar in size and shape to those resulting from mechanical stimulation at the same site. As might be expected with such a noxious stimulus, the nerve was usually damaged by this process. Gill preparations were more sensitive than skin, and responded within 2–3 sec to both ammonia and strong hydrochloric acid.

DISCUSSION

Are the recorded potentials of nervous origin?

Because it is unusual to be able to record nerve action potentials in the way described here, it is as well to review the evidence which suggests that they are in fact sensory nerve impulses.

First, on general grounds, the rapidly adapting response to mechanical stimulation is what might be predicted from a knowledge of other vertebrate cutaneous sensory nerves; the thermal and chemical sensitivities are also what might be expected. Secondly, the distribution of the recorded potentials over the skin of the ray agrees with the details of the cutaneous innervation observed histologically, and in particular the spatial separation of the different electrically-recorded units is similar to the anatomically-visible separation of the small bundles of fibres which run up through the stratum compactum of the dermis. Thirdly, the potentials are the same as those which can be recorded from the surface of the skin when the nerve at a distance is stimulated electrically and antidromic impulses arrive at the nerve endings. Fourthly, when simultaneous records are made of the potentials in skin and nerve, the 1:1 relationship between them indicates that it is the same impulse which is recorded at the two sites after propagation. It is most unlikely that the two records represent the same uniquely localized potential picked up by the two separate pathways because the difference in the duration would be most improbable. One can contrast the recording of the e.c.g. with various leads, where, although the pattern of potentials appears very different, it is only in the amplitude and sign that there are differences and never in duration. The time interval between the two records is discussed in more detail below.

Although there is some evidence that the potentials which have been recorded are the result of activity in more than one fibre, this does not alter the argument, since the synchronization between them is so exact. This question will be further discussed later.

If it is accepted that the recorded potentials represent the impulse activity in the cutaneous nerve fibres, there are a number of points which have to be explained. These include the polarity and shape of the individual potentials, their duration and latency, the long 'refractoriness' and the fact that they can be recorded at all without dissection or the use of micro-electrodes.

The characteristics and site of initiation of the potentials

It will be convenient at this point to indicate briefly the hypothesis to be proposed: it is suggested that the effect of the mechanical stimulus on the nerve endings is transmitted by a means not experimentally detectable to a point some distance away on the stem axon where an action potential is initiated. From this point impulses are propagated both centrally towards the C.N.S. and also back towards the skin, and it is the latter antidromically-directed impulses which are recorded by the electrode on the surface.

The recorded potentials are in general diphasic. There are two possible causes of this: either there is a potential of the same sign affecting first one electrode and then the other, or alternatively the electrical events are all at the same site, but consist of an action potential with a large after-hyperpolarization. The latter explanation is unlikely because of the rapidity and size of the negative phase in many of the records; it is also improbable than an after-hyperpolarization would be as large as three-quarters of the amplitude of the action potential, and yet nearly half the records have as much or more than this. On the other hand the long duration of the negative phase does not look like a normal action potential and the interval between the peaks of the positive and the negative phases is far longer than is found in the diphasic potentials of normal axons. But of course the nerve endings are not normal axons, and it is justifiable to look for similarities among other structures where impulses are initiated, for example in the frog muscle receptor, where the prepotentials in the fine terminal branches of the sensory nerve have a relatively long time course (Katz, 1950), and in central nervous dendrites where the time relationships of impulses (when they occur) are much longer (up to 15 msec) than in normal axons (Purpura, 1959).

In discussing the reasons for the polarity of the recorded potentials it will be assumed that they are normal negative spikes and that it is the spatial relationship of the nerve and electrodes that causes the first phase to be recorded as positive. For example, an electrode on the cut end of a nerve records monophasic impulses as positive relative to an indifferent electrode (e.g. Murray, 1956), and in the same way here the electrode on the surface may be 'seeing into the inside' of the nerve. In the case of a nerve

lying in a volume conductor of relatively high resistance, with one electrode on the nerve and the other at a distance, the electrode on the nerve will detect an approaching or receding impulse as a positive-going potential, and only when the impulse arrives will it be recorded as negative. In practice, the activity of single units cannot be detected unless the nerve is arranged to pass across a high-resistance region between the electrodes, and this normally is provided by an air gap or paraffin. However, there is something corresponding to such an air gap in the skin, and that is the dense fibrous layer, the stratum compactum, in the dermis, through which small bundles of fibres pass. If this is the high-resistance region, then the positive phase will represent activity of the nerve on the deep side (the side further from the surface of the skin) and the negative phase activity at the surface side. Impulses in the nerve entirely on the deep side of the stratum compactum, or electrical changes in the nerve endings in the superficial dermal and epidermal region will not be recorded because they will be short-circuited by the relatively low-resistance tissue fluids.

In the experiments the skin must be allowed to dry before the potentials are recorded at full height; it seems therefore that a relatively high horizontal resistance of the superficial layers is essential, as well as the high resistance of the stratum compactum. But if the superficial layers have too high a resistance, as in mammals, nothing is recorded. The mucous secretion covering the skin in elasmobranchs is also important in allowing the potentials to be recorded, for it prevents complete desiccation. In the gill preparations, where the epidermis is much thinner, the skin is mechanically less robust and does not survive so well in experiments. The high electrical resistance of the skin may well be important naturally in connexion with the electrical sensitivity of the ampullae of Lorenzini (Murray, 1960).

Apart from detailed anatomical considerations of the exact localization of the site of impulse initiation in relation to the stratum compactum, the positive-negative sequence of polarities indicates that the impulse is approaching the electrode on the skin; the result of antidromic electrical stimulation which gives rise to potentials with the same positive-negative sequence shows that the impulse is approaching from the direction of the stem axon, so even with 'orthodromic' stimulation the actual recorded impulse is moving antidromically. This means that the first potential changes large enough to be recorded must occur at some distance from the nerve endings, a distance great enough to allow of antidromic propagation. The extent to which the action potential propagates back into the upper layers of the skin will determine the diphasic shape of the recorded potentials. It is reasonable to suppose that the site of action-potential initiation is near the deep side of the stratum compactum, at a point, for example,

300 μ or more from the end of the myelination. Further evidence for the distance from the nerve endings will be discussed later.

The results of the experiments in which galvanic polarization was used are relevant here. The fully documented and consistent experiments in which anodal polarization of the skin resulted in an increase of frequency, or initiation of impulses, together with a decrease in the size of the positive phase and increase in the negative, support the hypothesis of initiation of impulses at a distance from the ends of the fibres. One can assume that the impulses start from a region which is depolarized rather than hyperpolarized by the stimulating direction of current, and this cannot be the ends of the fibres for they are nearest to the anode. It is also reasonable to find that the height of the positive phase of the action potentials is reduced by such stimulating currents if the positive phase corresponds to the occurrence of the action potentials at the region and moment of initiation (see also Murray, 1956, for a similar argument).

The time relations between the potentials recorded from the gill skin and the pre-trematic nerve branch can now be discussed. Since antidromic impulses which have been initiated by electrical stimulation of the nerve take 1–2·5 msec to reach the skin, one might suppose that orthodromic impulses resulting from mechanical stimulation of the skin should be recorded 1–2·5 msec later from the nerve than from the skin. However, the two situations are not comparable, since the antidromic impulse has to travel the full distance between the electrode on the nerve and the skin, whereas the orthodromic impulse starts at some intermediate site, and propagates in both directions, both backwards to the skin and onwards in the nerve. It is only necessary to postulate that the short distance of antidromic propagation to the skin is covered at a slower rate than the longer orthodromic pathway to the electrode on the nerve, and the short interval between the two recorded potentials can be explained. The slower conduction in the terminal region is reasonable since it agrees with the longer duration of the potentials (three times longer, for example) and the longer interval between the peaks of the positive and negative phases. However, it may also be necessary to postulate that the site of impulse initiation is a little distance down the nerve and not directly under the stratum compactum. In the preparation which is illustrated in Fig. 12 the stimulus was located 1·5 mm distant from the recording electrode, and so it is possible that the impulse was started in a branch of the fibre too distant for the activity in its terminal region to be detected by the recording electrode. So impulses would propagate both centrally to the recording electrode on the branchial nerve and from the point of branching antidromically to the recording electrode on the skin.

An explanation of the double-peaked potentials can also be given. Such

compound potentials obviously result from the nearly simultaneous occurrence of simple diphasic potentials in two or more fibres, so what is needed is an explanation of the synchronization. When the double peak follows the onset of the mechanical stimulus it is clear that two or more fibres are being stimulated, because of the lack of all-or-none properties of the potential. If, by chance, the two impulses follow the stimulus with different latencies, they would combine to give the compound recorded potential. Altering the position of the stimulus alters the relative latencies and so changes the recorded shape. But when the double-peaked potentials are all-or-none, and form a repetitive series, the synchronization is more difficult to explain. The anatomical picture shows that there are at least two to three fibres running in each channel through the stratum compactum, and it is just possible that these fibres lie so closely together that the activity of one regularly triggers off the activity of another. But this explanation is less likely than that the double potentials are due to the branching of the fibre concerned deeper in the dermis, so that the impulse propagating centrally also returns to the skin in a collateral branch, possibly through an adjacent channel, with a resulting delay of about 1 msec (i.e. a small-scale axon reflex). The two branches would have to be close enough for their activity to be recorded by the same electrode. The fact that a change in the site of stimulation results in a change of shape of the compound potential can also be explained if the impulse comes to be initiated in the second rather than in the first branch, or in both at once. The triphasic potentials presumably result from the superposition of simpler potentials of different durations and shapes; it does not seem profitable to analyse them further at this stage.

The events at the nerve endings

There remain the related problems of how the events at the ends of the fibres resulting from the stimulus bring about initiation of all-or-nothing propagated impulses in the stem axon some 300 μ away, and of the long latency which this process involves. In the sequence of events: mechanical stimulus—depolarization of the nerve endings—depolarization of stem axon—impulse initiation, it is possible that the delay may occur at any stage; but although the hypothesis of chemical stimulation of the nerve endings by substances released by the surrounding epidermal cells could account for the observed latencies even after short stimuli it seems unnecessary in the present context, and evidence from other cutaneous receptors does not require it.

It will therefore be assumed that mechanical stimulation can produce almost instantaneously in the nerve endings a receptor potential (terminology as in Davis, 1961) which is graded in size and probably in extent

according to the strength of the stimulus. The simpler explanation is then merely that the terminal non-myelinated endings of the fibre are not capable of generating an action potential, or at least have a progressively higher threshold towards the ends. So the effect of the receptor potential at the tip spreads proximally by local currents as does applied electrotonus and, if necessary, by summation with the effects in the adjacent branches, causes a depolarization adequate for impulse initiation in the myelinated stem axon (i.e. the receptor potential is here also a generator potential). There would therefore be little effective delay between stimulus and the onset of the depolarization in the stem axon, and the latency must be accounted for in terms of slow depolarization such as Hodgkin (1948) found in the non-myelinated axons of the crab leg under d.c. stimulation (what he called the response time). The action potential, once initiated in a region where the threshold is low, would be an adequate stimulus to the less sensitive regions lying more distally, and the impulse would propagate some distance antidromically back towards the endings as well as propagating normally on towards the C.N.S. This would account for the negative phase of the recorded potentials.

This explanation is plausible, and does not require any mechanisms in the nerve ending other than those which are known to be present in sense organs such as the Pacinian corpuscle (Gray, 1959; Loewenstein, 1959). But it is hard to explain how the initiation of an impulse can regularly occur as long as 20 msec after the end of a short-duration mechanical stimulus unless the mechanism of the connexion between nerve ending and stem axon is a propagation and not just electrotonic spread, i.e. something which is triggered and is then self-maintaining, and not just dependent on what is happening at the end of the fibre. The dependence of latency on temperature is a further argument in favour of an active propagation.

If then there is a propagated prepotential travelling between the nerve ending and the region of the stem axon where full-sized action potentials are initiated, there must be some reason why it is not detected as it passes down through the stratum compactum, for it is the activity of this part of the nerve which gives rise to the negative phase of the diphasic potential. It has been assumed that a potential is recorded when there is a difference between the membrane potentials in a nerve fibre at each side of a high-resistance layer in the skin (presumably the stratum compactum); so the absence from the record of an indication of the prepotential can mean either that it is of too small an amplitude (but potentials one-twentieth of the size of the positive phase would have been identified in some of the preparations) or more likely that the prepotential is of relatively long duration and propagates slowly (the long latency suggests this in any case). The slowly rising and slowly moving prepotential would not then result in

a great difference between the two recording electrodes (small spike size and slow propagation are usually found together in other nerves).

The only other evidence for what is happening in the nerve endings comes from the variations of latency with temperature and stimulus intensity. The variations with temperature do not require any specific type of prepotential, but the variations with stimulus intensity may be due either to the fact that the prepotentials are graded in size according to the stimulus, and so in speed, or else that the stronger stimulus starts off the prepotentials from a point further from the end of the fibre, so that they have not so far to travel before they reach the site where the all-or-none potentials originate. The former possibility involves prepotentials of graded amplitude, which are not yet generally accepted as being a common property of nervous structures; however, there is some evidence that graded prepotentials may occur in the dendrites of central neurones (Purpura, 1959).

'Refractoriness'

The long 'refractoriness' of the preparations is unusual; however, Catton (1960) has described something superficially similar from frog skin, where the full sensitivity of the cutaneous endings does not return for up to 2 sec after an impulse has been initiated either by mechanical or electrical stimulation. But it does not seem that the two phenomena are comparable. In the present experiments in the ray, as in the frog (Loewenstein, 1956), it is probable that the adaptation of the impulse discharge to maintained mechanical stimulation is due to a process of 'accommodation' of the tissues, an actual movement away from the stimulus which takes a few seconds to occur, the time resulting from the physical properties of the tissues such as their viscosity and elasticity. Even though the stimulus appears to be maintained, yet the immediate mechanical effect on the nerve endings dies away. If this is so, the same physical processes would result in the observed phenomena of 'refractoriness'. On removal of a stimulus, the skin would take several seconds to revert to its original position, and therefore the sensitivity would take the same time to regain its original value. The main evidence for this explanation is the temperature-insensitivity of the 'refractoriness' and the similarity of the time course of its onset at the beginning of a repeated train of short stimuli, the time course of the recovery after a long test stimulus, and the time course of adaptation itself.

SUMMARY

1. Diphasic action potentials can be recorded from the endings of the cutaneous nerves of elasmobranch fishes with electrodes resting on the surface of the skin.

2. The potentials occur in response to mechanical, chemical, thermal and electrical stimulation. Rapid adaptation is shown to maintained mechanical stimuli.

3. The first phase of the diphasic potential is positive, recorded by the electrode on the skin relative to an indifferent electrode. The duration of the potentials is about four times longer than that of the action potentials in the axons of the same nerves. The duration is temperature-dependent, shorter when hot, $Q_{10} = 1{\cdot}6$.

4. The latency of initiation of the impulses varies inversely with both temperature ($Q_{10} = 2{\cdot}1$) and stimulus intensity. For threshold stimuli the maximum latencies range from 6 to 70 msec, and with stronger stimuli the latency is reduced to 2·2–20 msec.

5. Antidromic impulses, initiated by electrical stimulation of the branchial nerve and recorded by an electrode on the skin of the gill bar, have the same polarity and shape as the impulses similarly recorded but initiated by mechanical stimulation of the skin.

6. Impulses recorded from the surface of the gill bar and from the branchial nerve occur in a 1:1 relationship with each other. The potentials in the nerve are of shorter duration and start later.

7. The polarity and other properties of the action potentials are interpreted as strongly suggesting that full-sized propagated impulses are initiated at a point distant from the nerve endings, and travel both centrally and back into the terminal regions of the nerve.

8. A property similar to refractoriness is described, but it is attributed to accommodative changes in the tissues.

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